Differential Diagnosis of Colorectal Cancer and other Diseases of the Colon

The present invention provides biomolecules and the use of these biomolecules for the differential diagnosis of colorectal cancer or a non-malignant disease of the large intestine. In specific embodiments, the biomolecules are characterised by mass profiles generated by contacting a test and/or biological sample with an anion exchange surface under specific binding conditions and detecting said biomolecules using gas phase ion spectrometry. The biomolecules used according to the invention are preferably proteins or polypeptides. Furthermore, preferred test and/or biological samples are blood serum samples and are of human origin.

BACKGROUND TO THE INVENTION

Colorectal cancer is the fourth most common cancer in the world to date, and accounts for approximately 200,000 deaths per year in Europe and the US alone. Although colorectal cancer generally affects both men and women equally (currently at 9.4% and 10.1% of incident cancer, respectively), its distribution as a leading cause of death in men and women is disproportionate. Whereas colorectal cancer is the fourth leading cancer-related cause of death in men (following lung, stomach and prostate cancer), in women it takes second place to breast cancer. Furthermore, colorectal cancer is more prevalent in developed countries exhibiting more westernised lifestyle practices.

Familial and hereditary factors have been observed to play primary roles in the cause of colorectal cancers. In addition, a number of other factors have been shown to be associated with an increased risk of developing colorectal cancer namely the presence of adenomatous polyps, history/presence of inflammatory bowel disease, diets rich in animal fats and significantly decreased consumption of raw or fresh vegetables (especially leafy green vegetables, cruciferous vegetables, as well as allium vegetables such as garlic, onions, chives).

Significant differences exist regarding the survival of patients affected by colorectal cancer according to the stages at which the disease is diagnosed. Most patients exhibit symptoms such as rectal bleeding, pain, abdominal distension or weight loss only after the disease is in its advanced stages, leaving little therapeutic options available. Clearly, early detection of primary, metastatic, and recurrent disease can significantly impact the prognosis of individuals suffering from colorectal cancer. Diagnosis at an early stage, prior to lymph-node spread, can significantly improve the rate of survival as compared to a diagnosis established at a later stage of the disease, since the therapies used to treat colorectal cancer are stage-dependent.

To date, fecal occult blood test (FOBT), flexible sigmoidoscopy, double contrast barium enema, and colonoscopy are the primary tools utilised to detect colorectal cancer at its early stages. Among these

only FOBT, which is based on the high probability that blood found within a patients' fecal (heme-positive) sample arises from tumours found within the large intestine, is non-invasive, simple and relatively inexpensive. Unfortunately, this method of early detection has several drawbacks.

Firstly, a positive FOBT result leads to further examination, mainly colonoscopy -- an extremely discomforting, invasive diagnostic method which is expensive and carries a serious complication rate of one per 5,000 examinations. Colonoscopy, as a follow-up diagnostic method, might prove to be effective in confirming colorectal cancer within a patient provided that the FOBT results indeed reflect the presence of the disease. Unfortunately this is more often not the case, since only 12% of the patients with a heme-positive fecal sample are diagnosed with cancer or large polyps at the time of colonoscopy. Furthermore, physicians frequently fail to properly instruct their patients on how fecal samples should be collected. Normally, patients are told to adhere to specific dietary guidelines and to avoid taking medication known to induce gastrointestinal bleeding. Should the patient not be instructed properly, nor adhere to the strict protocol, the chance of obtaining a false-positive FOBT result is greatly increased. The false positive-FOBT result will subsequently send the patient for a confirmatory diagnosis, which is neither necessary, inexpensive, or pleasant. Secondly, a false-negative result holds even greater consequence since a patient possessing colorectal cancer, in this case, would not be diagnosed as having the disease and would be sent home without proper therapy.

Currently, many groups are utilising proteomic technologies to comparatively analyse the differences in protein levels in colorectal cancers vs. normal large intestinal tissue in the hopes of developing diagnostic markers that could assist the practicing clinician in the management of colorectal cancer. Currently, the standard method of proteome analysis has been two dimensional (2D) gel electrophoresis, which has been an invaluable tool for the separation and identification of proteins. This method is also effective in identifying aberrantly expressed proteins in a variety of tissue samples. Unfortunately, the analysis of data generated by 2D-gel electrophoresis is labour-intensive and requires large quantities of material for protein analysis, thereby rendering it impractical for routine clinical use.

Through the introduction of SELDI (surface enhanced laser desorption ionization), a modification of MALDI-TOF (matrix-assisted laser desorption ionization/time of flight) which is a mass spectrometry technique that allows for the simultaneous analysis of multiple proteins in one sample, this tool has been achieved. Small amounts of proteins can be directly bound to a biochip, carrying spots with different types of chromatographic material, including those with hydrophobic, hydrophilic, cation-exchanging and anion-exchanging characteristics. This approach has been proven to be very useful to identify proteins and protein patterns (profiles) in various biological fluids, including serum, urine or

pancreatic juice.

To date, specific biomarkers for the detection of breast and prostate cancers (patents WO0223200, WO03058198 and WO0125791 from Ciphergen, respectively) have been identified using the above mentioned SELDI technology. Unfortunately, due to the nature of sample testing, the biomarkers identified can only be used to diagnose a patient as having a specific cancer (either breast or prostate) versus not having the disease at all. For example, the sera samples analyzed in WO03058198 (Ciphergen) and WO0223200 (Ciphergen) were taken from patients with late-stage breast cancer (stages III and IV), the control samples were taken from patients with undetectable breast cancer. The biomarkers identified are neither grade-specific nor can they detect the disease at its earliest stages (stage I and II), and thereby would not allow for effective patient-specific treatment of the disease. Moreover, biomarkers that can differentiate between the presence of a colorectal cancer, a non-malignant disease of the large intestine, or an acute and chronic inflammation of the epithelium have not yet been identified.

Accordingly, there is a critical need to develop a simple, non-invasive, reliable and inexpensive method for the effective detection of colorectal cancer at its early stages. Preferably, such a diagnostic method should be able to detect early-stage colorectal cancers as well as distinguish between the later stages or grades of the disease. With such valuable information, medical practitioners would be able to tailor patient therapies for optimum treatment of the disease.

The present invention addresses this difficulty with the development of a non-invasive diagnostic tool for the differential diagnosis of colorectal cancer and non-malignant diseases of the large intestine.

SUMMARY OF THE INVENTION

The present invention relates to methods for the differential diagnosis of colorectal cancer or non-malignant disease of the large intestine by detecting one or more differentially expressed biomolecules within a test sample of a given subject, comparing results with samples from healthy subjects, subjects having a precancerous lesion of the large intestine, subjects having a colorectal cancer, subjects having a metastasised colorectal cancer, or subjects having a non-malignant disease of the large intestine, wherein the comparison allows for the differential diagnosis of a subject as healthy, having a precancerous lesion of the large intestine, having a colorectal cancer, having a metastasised colorectal cancer or a non-malignant disease of the large intestine.

The present invention provides a method for the differential diagnosis of a colorectal cancer and/or a non-malignant disease of the large intestine, *in vitro*, comprising obtaining a test sample from a subject, contacting test sample with a biologically active surface under specific binding conditions,

allowing for biomolecules present within the test sample to bind to the biologically active surface, detecting one or more bound biomolecules using mass spectrometry thereby generating a mass profile of said test sample, transforming data into a computer-readable form, and comparing said mass profile against a database containing mass profiles specific for healthy subjects, subjects having a precancerous lesion of the large intestine, subjects having colorectal cancer, subjects having metastasised colorectal cancers, or subjects having a non-malignant disease of the large intestine, wherein the comparison allows for the differential diagnosis of a subject as healthy, having a precancerous lesion of the large intestine, having a colorectal cancer, having a metastasised colorectal cancer or a non-malignant disease of the large intestine.

In one embodiment the invention provides a database comprising of mass profiles of biological samples from healthy subjects, subjects having a precancerous lesion of the large intestine, subjects having a colorectal cancer, subjects having a metastasised colorectal cancer, or subjects having a non-malignant disease of the large intestine.

Within the same embodiment the database is generated by obtaining biological samples from healthy subjects, subjects having a precancerous lesion of the large intestine, subjects having a colorectal cancer, subjects having a metastasised colorectal cancer, and subjects having a non-malignant disease of the large intestine, contacting said biological samples with a biologically active surface under specific binding conditions, allowing the biomolecules within the biological sample to bind to said biologically active surface, detecting one or more bound biomolecules using mass spectrometry thereby generating a mass profile of said biological samples, transforming data into a computer-readable form, and applying a mathematical algorithm to classify the mass profiles as specific for healthy subjects, subjects having a precancerous lesion of the large intestine, subjects having colorectal cancer, subjects having metastasised colorectal cancer, and subjects having a non-malignant disease of the large intestine.

In specific embodiments, the present invention provides biomolecules having a molecular mass selected from the group consisting of 2020 Da ± 10 Da, 2049 Da ± 10 Da, 2270 Da ± 11 Da, 2508 Da ± 13 Da, 2732 Da ± 14 Da, 3026 Da ± 15 Da, 3227 Da ± 17 Da, 3326 Da ± 17 Da, 3436 Da ± 17 Da, 3946 Da ± 20 Da, 4103 Da ± 21 Da, 4243 Da ± 21 Da, 4305 Da ± 21 Da, 4359 Da ± 22 Da, 4476 Da ± 22 Da, 4546 Da ± 23 Da, 4607 Da ± 23 Da, 4719 Da ± 24 Da, 4830 Da ± 24 Da, 4865 Da ± 24 Da, 4965 Da ± 25 Da, 5112 Da ± 26 Da, 5226 Da ± 26 Da, 5493 Da ± 27 Da, 5646 Da ± 28 Da, 5772 Da ± 29 Da, 5854 Da ± 29 Da, 6446 Da ± 32 Da, 6644 Da ± 33 Da, 6852 Da ± 34 Da, 6897 Da ± 34 Da, 6999 Da ± 35 Da, 7575 Da ± 38 Da, 7657 Da ± 38 Da, 8076 Da ± 40 Da, 8215 Da ± 41 Da, 8474 Da ± 42 Da, 8574 Da ± 43 Da, 8702 Da ± 44 Da, 8780 Da ± 44 Da, 8922 Da ± 45 Da, 9078 Da ± 45 Da, 9143 Da ± 46 Da, 9201 Da ± 46 Da, 9359 Da ± 47 Da, 9425 Da ± 47 Da, 9581 Da ± 48 Da, 9641 Da ±

43 Da, 9718 Da ± 49 Da, 9930 Da ± 50 Da, 10215 Da ± 51 Da, 10369 Da ± 52 Da, 10440 Da ± 52 Da, 10594 Da ± 53 Da, 11216 Da ± 56 Da, 11464 Da ± 57 Da, 11547 Da ± 58 Da, 11693 Da ± 58 Da, 11905 Da ± 60 Da, 12470 Da ± 62 Da, 12619 Da ± 63 Da, 12828 Da ± 64 Da, 13290 Da ± 66 Da, 13532 Da ± 68 Da, 13784 Da ± 69 Da, 13983 Da ± 70 Da, 14798 Da ± 74 Da, 15065 Da ± 75 Da, 15140 Da ± 76 Da, 15350 Da ± 77 Da, 15879 Da ± 79 Da, 15957 Da ± 80 Da, 16104 Da ± 81 Da, 16164 Da ± 81 Da, 16953 Da ± 85 Da, 17263 Da ± 86 Da, 17597 Da ± 87 Da, 17617 Da ± 88 Da, 17765 Da ± 89 Da, 17890 Da ± 89 Da, 18115 Da ± 91 Da, 18398 Da ± 92 Da, 22358 Da ± 112 Da, 22466 Da ± 112 Da, 22676 Da ± 113 Da, 22951 Da ± 115 Da, 24079 Da ± 120 Da, 28053 Da ± 140 Da, and 28259 Da ± 141 Da. The biomolecules having said molecular masses are detected by contacting a test and/or biological sample with a biologically active surface comprising an adsorbent under specific binding conditions and further analyzed by gas phase ion spectrometry. Preferably the adsorbent used is comprised of positively charged quaternary ammonium groups (anion exchange surface).

In specific embodiments, the invention provides specific binding conditions for the detection of biomolecules within a sample. In preferred embodiments, a sample is diluted 1:5 in a denaturation buffer, consisting of 9 M urea, 2 % glycerol, 4% CHAPS, 10x DTT, 4x pH 4 ampholine, and then diluted again 1:10 in binding buffer consisting of 0.1 M Tris-HCl, 0.05% Triton X-100 at a pH 8.5 to 9.0 to 4°C. The diluted sample is then contacted with a biologically active surface comprising of positively charged (cationic) quaternary ammonium groups (anion exchange), incubated for 120 minutes at 20 to 24°C and the bound biomolecules are detected using gas phase ion spectrometry.

In an alternative embodiment, the invention provides a method for the differential diagnosis of a colorectal cancer and/or a non-malignant disease of the large intestine comprising detecting of one or more differentially expressed biomolecules within a sample. This method comprises obtaining a test sample from a subject, contacting said sample with a binding molecule specific for a differentially expressed polypeptide, allowing an interaction between the binding molecule and the specific polypeptide, wherein the interaction of an interaction indicates the presence or absence of said polypeptide thereby allowing for the differential diagnosis of a subject as healthy, having a precancerous polyp of the large intestine, having a colorectal cancer, having a malignant colorectal cancer, and/or a non-malignant disease of the large intestine. Preferably, binding molecules are antibodies specific for said polypeptides.

The biomolecules related to the invention, having a molecular mass selected from the group consisting of 2020 Da ± 10 Da, 2049 Da ± 10 Da, 2870 Da ± 11 Da, 2501 Da ± 13 Da, 2732 Da ± 14 Da, 3025 Da ± 15 Da, 3222 Da ± 17 Da, 3326 Da ± 17 Da, 3456 Da ± 17 Da, 3546 Da ± 20 Da, 4103 Da ± 21 Da, 4141 Da ± 21 Da, 4295 Da ± 21 Da, 4359 Da ± 22 Da, 4476 Da ± 23 Da, 4548 Da ± 23 Da, 4607

Da ± 23 Da, 4719 Da ± 24 Da, 4830 Da ± 24 Da, 4865 Da ± 24 Da, 4963 Da ± 25 Da, 5112 Da ± 26 Da, 5226 Da ± 26 Da, 5409 Da ± 27 Da, 5648 Da ± 28 Da, 5772 Da ± 29 Da, 5854 Da ± 29 Da, 6446 Da ± 32 Da, 6644 Da ± 33 Da, 6852 Da ± 34 Da, 6897 Da ± 34 Da, 6999 Da ± 35 Da, 7575 Da ± 38 Da, 7657 Da ± 38 Da, 8076 Da ± 40 Da, 8215 Da ± 41 Da, 8474 Da ± 42 Da, 8574 Da ± 43 Da, 8702 Da ± 44 Da, 8780 Da ± 44 Da, 8922 Da ± 45 Da, 9078 Da ± 45 Da, 9143 Da ± 46 Da, 9201 Da ± 46 Da, 9351 Da ± 47 Da, 9423 Da ± 47 Da, 9581 Da ± 48 Da, 9641 Da ± 48 Da, 9718 Da ± 49 Da, 9930 Da ± 50 Da, 10215 Da ± 51 Da, 10369 Da ± 52 Da, 10440 Da ± 52 Da, 10594 Da ± 53 Da, 11216 Da ± 56 Da, 11464 Da ± 57 Da, 11547 Da ± 58 Da, 11693 Da ± 58 Da, 11905 Da ± 60 Da, 12470 Da ± 62 Da, 12619 Da ± 63 Da, 12828 Da ± 64 Da, 13290 Da ± 66 Da, 13532 Da ± 68 Da, 13784 Da ± 69 Da, 13983 Da ± 70 Da, 14798 Da ± 74 Da, 15065 Da ± 75 Da, 15140 Da ± 76 Da, 15350 Da ± 77 Da, 15879 Da ± 79 Da, 15957 Da ± 80 Da, 16104 Da ± 81 Da, 16164 Da ± 81 Da, 16953 Da ± 85 Da, 17263 Da ± 86 Da, 17597 Da ± 87 Da, 17617 Da ± 88 Da, 17765 Da ± 89 Da, 17890 Da ± 89 Da, 18115 Da ± 91 Da, 18398 Da ± 92 Da, 22358 Da ± 112 Da, 22466 Da ± 112 Da, 22676 Da ± 113 Da, 22951 Da ± 115 Da, 24079 Da ± 120 Da, 28053 Da ± 140 Da, or 28259 Da ± 141 Da, and may include, but are not limited to, molecules comprising nucleotides, amino acids, sugars, fatty acids, steroids, nucleic acids, polynucleotides (DNA or RNA), polypeptides, proteins, antibodies, carbohydrates, lipids, and combinations thereof (e.g., glycoproteins, ribonucleoproteins, lipoproteins). Preferably said biomolecules are proteins, polypeptides, or fragments thereof.

In yet another embodiment, the invention provides a method for the identification of biomolecules within a sample, provided that the biomolecules are proteins, polypeptides or fragments thereof, comprising chromatography and fractionation, analysis of fractions for the presence of said differentially expressed proteins and/or fragments thereof, using a biologically active surface, further analysis using mass spectrometry to obtain amino acid sequences encoding said protein and/or fragments thereof, and searching amino acid sequence databases of known proteins to identify said differentially expressed proteins by amino acid sequence comparison. Preferably the method of chromatography is high performance liquid chromatography (HPLC) or fast protein liquid chromatography (FPLC). Furthermore, the mass spectrometry used is selected from the group of matrix-assisted laser desorption ionization/time of flight (MALDI-TOF), surface enhanced laser desorption ionization/time of flight (SELDI-TOF), liquid chromatography, MS-MS, or ESI-MS.

Furthermore, the invention provides kits for the differential diagnosis of a colorectal cancer and/or a non-malignant disease of the colon.

The test or biological samples used according to the invention may be of blood, blood serum, plasma, nipple aspirate, urine, semen, seminal fluid, seminal plasma, prostatic fluid, excreta, feces, saliva, sweat, biopsy, ascites, cerebrospinal fluid, milk, lymph, or tissue extract origin. Preferably, the test

and/or biological samples are blood serum samples, and are isolated from subjects of mammalian origin, preferably of human origin.

A colorectal cancer of the invention is a cancer of the large intestine, and may include cancers of the colon, rectum etc. Furthermore, a colorectal cancer, as intended by the invention, may be of various stages and/or grades.

DESCRIPTION OF FIGURES

Figure 1. Comparison of protein mass spectra processed on the anion exchange surface of a SAX2 ProteinChip array comprised of cationic quaternary ammonium groups. Protein mass spectra obtained from sera of endoscopy control patients (C1 and C2), suffering from non-malignant diseases of the large intestine (e.g., acute or chronic inflammation, adenoma) and of patients with colon cancer (T1 and T2) are shown. Scattered boxes indicate differentially expressed proteins with high diagnostic significance. A representative differentially expressed protein (m/z= 6645 Da) is highlighted possessing high importance within the generated classifiers (ensemble of decision trees) according to overall improvement, see Tables 1-4. The X-axis shows the mass/charge (m/z) ratio, which is equivalent to the apparent molecular mass of the corresponding biomolecule. The Y-axis shows the normalized relative signal intensity of the peak in the examined serum samples.

Figure 2A - F. Scatter plots of clusters (peaks, variables), belonging to differentially expressed proteins included in the four classifiers. The X-axis shows the mass/charge (m/z) ratio, which is equivalent to the apparent molecular mass of the corresponding biomolecule. The Y-axis shows the logarithmic normalized relative signal intensity of the peaks in the examined serum samples. First, intensities were shifted to yield entirely positive values. Then, for each mass, intensities were normalized by dividing the intensity values by the average intensity of that mass. Finally, the natural logarithm was taken. □ T (Tumour): Colon cancer patients' serum samples. ○ N (Normal): Endoscopy control patients' serum samples.

Figure 3A - F. Additionally scaled scatter plots of clusters (peaks, variables), belonging to differentially expressed proteins included in the four classifiers. The X-axis shows the mass/charge (m/z) ratio, which is equivalent to the apparent molecular mass of the corresponding biomolecule. As in Figure 2, the Y-axis shows the logarithmic normalized relative signal intensity of the peaks in the examined serum samples. However, intensities were additionally (shifted and) scaled so that the intensities of each mass cover the entire range of the Y-axis. Thereby, the minimum and maximum intensities of all masses are aligned on the lower and upper edge of the plot, respectively. This allows to better visualize the extend of class overlap. □ T (Tumour): Colon cancer patients' serum samples. ○ N (Normal): Endoscopy control patients' serum samples.

Figure 4. Complexity of proof-of-principle classifier. The histogram visualizes the distribution of the number of decision tree variables (peaks, clusters) for the obtained proof-of-principle classifier for gastric cancer. 6 variables per decision tree are typical.

Figure 5. Variable importance of the proof-of-principle classifier. The histograms visualize how often a variable (mass) is employed in the proof-of-principle classifier. The frequency of variable selection is presented in histogram form for each hierarchical level (a-j) and for all hierarchical levels taken together (k).

Figure 6. Complexity of 1st final classifier. The histogram visualizes the distribution of the number of decision tree variables (peaks, clusters) for the obtained 1st final classifier in the range of 1 to 10 decision tree variables. 9 variables per decision tree are typical.

Figure 7. Variable importance of 1st final classifier. The histogram visualizes how often a variable (mass) is employed in the final classifier. The frequency of variable selection is presented in histogram form for each of the first 10 hierarchical levels (a-j) and for the first ten hierarchical levels taken together (k).

Figure 8. Complexity of 2nd final classifier. The histogram visualizes the distribution of the number of decision tree variables (peaks, clusters) for the obtained 2nd final classifier in the range of 1 to 10 decision tree variables. As many as 10 variables per decision tree are typical.

Figure 9. Variable importance of 2nd final classifier. The histogram visualizes how often a variable (mass) is employed in the 2nd final classifier. The frequency of variable selection is presented in histogram form for each of the first 10 hierarchical levels (a-j) and for the first ten hierarchical levels taken together (k).

Figure 10. Complexity of 3rd final classifier. The histogram visualizes the distribution of the number of decision tree variables (peaks, clusters) for the obtained 3rd final classifier in the range of 1 to 10 decision tree variables. As many as 10 variables per decision tree are typical.

Figure 11. Variable importance of 3rd final classifier. The histogram visualizes how often a variable (mass) is employed in the 3rd final classifier. The frequency of variable selection is presented in histogram form for each of the first 10 hierarchical levels (a-j) and for the first ten hierarchical levels taken together (k).

DESCRIPTION OF THE INVENTION

It is to be understood that the present invention is not limited to the particular materials and methods described or equipment, as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which will be limited only by the appended claims.

It should be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "the antibody" is a reference to one or more antibodies and derivatives thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art. Although any materials and methods, or equipment [illegible] similar or equivalent to those described herein can be used in practice or test the present invention, the preferred equipment, materials and methods are described below. All publications mentioned herein are cited for the purpose of describing and disclosing protocols, reagents, and current state of the art [illegible] that might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Definitions

The term "biomolecule" refers to a molecule produced by a cell or living organism. Such molecules include, but are not limited to, molecules comprising nucleotides, amino acids, sugars, fatty acids, steroids, nucleic acids, polynucleotides, polypeptides, proteins, carbohydrates, lipids, and combinations thereof (e.g. glycoproteins, ribonucleoproteins, lipoproteins). Furthermore, the terms "nucleotide or polynucleotide" refer to a nucleotide, oligonucleotide, polynucleotide, or any fragment thereof. These phrases also refer to DNA or RNA of genomic or synthetic origin which may be single-stranded or double-stranded and may represent the sense or the antisense strand, to peptide polynucleotide sequences (i.e. peptide nucleic acids; PNAs), or to any DNA-like or RNA-like material.

The term "fragment" refers to a portion of a polypeptide (parent) sequence that comprises at least 10 consecutive amino acid residues and retains a biological activity and/or some functional characteristics of the parent polypeptide e.g. antigenicity or structural domain characteristics.

The terms "biological sample" and "test sample" refer to all biological fluids and excretions isolated from any given subject. In the context of the invention such samples include, but are not limited to,

blood, blood serum, plasma, nipple aspirate, urine, semen, seminal fluid, seminal plasma, prostatic fluid, excreta, tears, saliva, sweat, biopsy, ascites, cerebrospinal fluid, milk, lymph, or tissue extract samples.

The term "specific binding" refers to the binding reaction between a biomolecule and a specific "binding molecule". Related to the invention are binding molecules that include, but are not limited to; proteins, peptides, nucleotides, nucleic acids, hormones, amino acids, sugars, fatty acids, steroids, polynucleotides, carbohydrates, lipids, or a combination thereof (e.g. glycoproteins, ribonucleoproteins, lipoproteins). Furthermore, a binding reaction is considered to be specific when the interaction between said molecules is substantial. In the context of the invention, a binding reaction is considered substantial when the reaction that takes place between said molecules is at least two times the background. Moreover, the term "specific binding conditions" refers to reaction conditions that permit the binding of said molecules such as pH, salt, detergent and other conditions known to those skilled in the art.

The term "interaction" relates to the direct or indirect binding or alteration of biological activity of a biomolecule.

The term "differential diagnosis" refers to a diagnostic decision between a healthy and different disease status, including various stages of a specific disease. A subject is diagnosed as healthy or to be suffering from a specific disease, or a specific stage of a disease based on a set of hypotheses that allow for the distinction between healthy and one or more stages of the disease. The choice between healthy and one or more stages of disease depends on a significant difference between each hypothesis. Under the same principle, a "differential diagnosis" may also refer to a diagnostic decision between one disease type as compared to another (e.g. colon cancer vs. diverticulosis).

The term "colorectal cancer" refers to a cancer state associated with the large intestine of any given subject, wherein the cancer state is defined according to its stage and/or grade. The various stages of a cancer may be identified using staging systems known to those skilled in the art [e.g. Union Internationale Contre Cancer (UICC) system or American Joint Committee on Cancer (AJC)]. In the context of the invention colorectal cancers include but are not limited to colon and rectal cancers.

The term "non-malignant disease of the large intestine" refers to alterations in the physiological, functional and/or anatomical state of the large intestine, wherein the alterations deviate from normal. In addition, this term encompasses alterations in the physiological, functional and/or anatomical state of the large intestine that cannot be staged or graded according to cancer staging systems known to those skilled in the art [e.g. Union Internationale Contre Cancer (UICC) system or American Joint

Committee on Cancer (AJCC)]. Such non-malignant diseases include but are not limited to the acute and chronic inflammation of the large intestinal epithelium, diverticular disease including diverticulosis and diverticulitis, colitis, ulcerative colitis, proctitis, Crohn's disease (ileitis), pruritis, intestinal polyps including hyperplastic polyps, hamartomatous polyps (i.e. Juvenile polyps, Peutz-Jeghers polyps), inflammatory polyps, and lymphoid polyps, adenomatous polyps.

The term "healthy individual" refers to a subject possessing good health. Such a subject demonstrates an absence of any disease within the large intestine, preferably a colorectal cancer or a non-malignant disease of the large intestine.

The term "precancerous lesion of the large intestine" refers to a biological change within a cell and/or tissue of the large intestine such that said cell and/or tissue becomes susceptible to the development of a cancer. More specifically, a precancerous lesion of the large intestine is a preliminary stage of a colorectal cancer (i.e. dysplasia). Causes of a precancerous lesion of the large intestine may include, but are not limited to, genetic predisposition and exposure to cancer-causing agents (carcinogens), such cancer causing agents include agents that cause genetic damage and induce neoplastic transformation of a cell. Furthermore, the phrase "neoplastic transformation of a cell" refers to alterations in normal cell physiology and includes, but is not limited to, self-sufficiency in growth signals, insensitivity to growth-inhibitory (anti-growth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis.

The term "dysplasia" refers to morphological alterations within a tissue, which are characterized by a loss in the uniformity of individual cells, as well as a loss in their architectural orientation. Furthermore, dysplastic cells also exhibit a variation in size and shape.

The phrase "differentially present" refers to differences in the quantity of a biomolecule (of a particular sequence/molecular mass) present in a sample from a subject as compared to a comparable sample. For example, a biomolecule is present at an elevated level, a decreased level or absent in samples of subjects having colorectal cancer compared to samples of subjects who do not have a cancer of the large intestine. Therefore in the context of the invention, the term "differentially present biomolecule" refers to a biomolecule (of a particular sequence/molecular mass) present within a sample taken from a subject having a disease or cancer of the large intestine as compared to a comparable sample taken from a healthy subject. Within the context of the invention, a biomolecule is differentially present between two samples if the quantity of said biomolecule in one sample is statistically significantly different from the quantity of said biomolecule in another sample.

The term "diagnostic assay" can be used interchangeably with "diagnostic method" and refers to the

detection of the presence or nature of a pathologic condition. Diagnostic assays differ in their sensitivity and specificity. Within the context of the invention the sensitivity of a diagnostic assay is defined as the percentage of diseased subjects who test positive for a colorectal cancer or a non-malignant disease of the large intestine and are considered "true positives". Subjects having a colorectal cancer or a non-malignant disease of the large intestine but not detected by the diagnostic assay are considered "false negatives". Subjects who are not diseased and who test negative in the diagnostic assay are considered "true negatives". Furthermore, the term specificity of a diagnostic assay, as used herein, is defined as 1 minus the false positive rate, where the "false positive rate" is defined as the proportion of those subjects devoid of a colorectal cancer or a non-malignant disease of the large intestine but who test positive in said assay.

The term "adsorbent" refers to any material that is capable of accumulating (binding) a biomolecule. The adsorbent typically posts a biologically active surface and is composed of a single material or a plurality of different materials that are capable of binding a biomolecule. Such materials include, but are not limited to, anion exchange materials, cation exchange materials, metal chelators, polynucleotides, oligonucleotides, peptides, antibodies, metal chelators etc.

The term "biologically active surface" refers to any two- or three-dimensional extension of a material that biomolecules can bind to, or interact with, due to the specific biochemical properties of this material and those of the biomolecules. Such biochemical properties include, but are not limited to, ionic character (charge), hydrophobicity, or hydrophilicity.

The term "binding molecule" refers to a molecule that displays an affinity for another molecule. Within the context of the invention such molecules may include, but are not limited to nucleotides, amino acids, sugars, fatty acids, steroids, nucleic acids, polypeptides, carbohydrates, lipids, and combinations thereof (e.g. glycoproteins, ribonucleoproteins, lipoproteins). Preferably, such binding molecules are antibodies.

The term "solution" refers to a homogeneous mixture of two or more substances. Solutions may include, but are not limited to buffers, substrate solutions, elution solutions, wash solutions, detection solutions, desorption solutions, chemical solutions, solvents, etc. Furthermore, other solutions known to those skilled in the art are also included herein.

The term "mass profile" refers to a mass spectrum as a characteristic property of a given sample or a group of samples, especially when compared to the mass profile of a second sample or group of samples in any way different from the first sample or group of sample. In the context of the invention, the mass profile is obtained by treating the biological sample as follows. The sample is diluted 1:5 in

a denaturing buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, and 2% ampholine and subsequently diluted 1:10 in binding buffer consisting of 0.1 M Tris-HCl, 0.02% Triton X-100 at pH 8.5. This pre-treated sample is applied to a biologically active surface comprising positively charged quaternary ammonium groups (anion exchange surface) and incubated for 120 minutes. The biomolecules bound to the surface are analysed by gas phase ion spectrometry as described in another section. All but the diluting steps are performed at 20 to 24°C. Dilution steps are performed at 0 to 4°C.

The phrase "apparent molecular mass" refers to the molecular mass value in Dalton (Da) of a biomolecule as it may appear in a given method of investigation, e.g. size exclusion chromatography, gel electrophoresis, or mass spectrometry.

The term "chromatography" refers to any method of separating biomolecules within a given sample such that the original native state of a given biomolecule is retained. Separation of a biomolecule from other biomolecules within a given sample for the purpose of enrichment, purification and/or analysis, may be achieved by methods including, but not limited to, size exclusion chromatography, ion exchange chromatography, hydrophobic and hydrophilic interaction chromatography, metal affinity chromatography, wherein "metal" refers to metal ions (e.g. nickel, copper, gallium, or zinc) of all chemically possible valences, or ligand affinity chromatography wherein "ligand" refers to binding molecules, preferably proteins, antibodies, or DNA. Generally, chromatography uses biologically active surfaces as adsorbents to selectively accumulate certain biomolecules.

The term "mass spectrometry" refers to a method comprising employing an ionization source to generate gas phase ions from a biological entity of a sample presented on a biologically active surface and detecting the gas phase ions with a mass spectrometer.

The phrase "laser desorption mass spectrometry" refers to a method comprising the use of a laser as an ionization source to generate gas phase ions from a biomolecule presented on a biologically active surface and detecting the gas phase ions with a mass spectrometer.

The term "mass spectrometer" refers to a gas phase ion spectrometer that includes an inlet system, an ionization source, an ion optic assembly, a mass analyser, and a detector.

Within the context of the invention, the terms "detect", "detection" or "detecting" refer to the identification of the presence, absence, or quantity of a biomolecule.

The term "energy absorbing molecule" or "EAM" refers to a molecule that absorbs energy from an

energy source in a mass spectrometer thereby enabling desorption of a biomolecule from a biologically active surface. Cinnamic acid derivatives, sinapinic acid and dihydroxybenzoic acid are frequently used as energy-absorbing molecules in laser desorption of biomolecules. See U.S. Pat. No. 5,719,060 (Hutchens & Yip) for a further description of energy absorbing molecules.

The term "training set" refers to a subset of the respective entire available data set. This subset is typically randomly selected, and is solely used for the purpose of classifier construction.

The term "test set" refers to a subset of the entire available data set consisting of those entries not included in the training set. Test data is applied to evaluate classifier performance.

The term "decision tree" refers to a flow-chart-like tree structure employed for classification. Decision trees consist of repeated splits of a data set into subsets. Each split consists of a simple rule applied to one variable, e.g., "if value of 'variable 1' larger than 'threshold 1' then go left else go right". Accordingly, the given feature space is partitioned into a set of rectangles with each rectangle assigned to one class.

The terms "ensemble", "tree ensemble" or "ensemble classifier" can be used interchangeably and refer to a classifier that consists of many simpler elementary classifiers, e.g., an ensemble of decision trees is a classifier consisting of decision trees. The result of the ensemble classifier is obtained by combining all the results of its constituent classifiers, e.g., by majority voting that weights all constituent classifiers equally. Majority voting is especially reasonable in the case of bagging, where constituent classifiers are then naturally weighted by the frequency with which they are generated.

The term "competitor" refers to a variable (in our case: mass) that can be used as an alternative splitting rule in a decision tree. In each step of decision tree construction, only the variable yielding best data splitting is selected. Competitors are non-selected variables with similar but lower performance than the selected variable. They point into the direction of alternative decision trees.

The term "surrogate" refers to a splitting rule that closely mimics the action of the primary split. A surrogate is a variable that can substitute a selected decision tree variable, e.g. in the case of missing values. Not only must a good surrogate split the parent node into descendant nodes similar in size and composition to the primary descendant nodes. In addition, the surrogate must also match the primary split on the specific cases that go to the left child and right child nodes.

The terms "peak" and "signal" may be used interchangeably and refer to any signal which is generated by a biomolecule when under investigation using a specific method, for example chromatography,

mass spectrometry, or any type of spectroscopy like Ultraviolet/Visible Light (UV/Vis) spectroscopy, Fourier Transformed Infrared (FTIR) spectroscopy, Electron Paramagnetic Resonance (EPR) spectroscopy, or Nuclear Magnet Resonance (NMR) spectroscopy.

Within the context of the invention, the terms "peak" and "signal" refer to the signal generated by a biomolecule of a certain molecular mass hitting the detector of a mass spectrometer, thus generating a signal intensity which correlates with the amount or concentration of said biomolecule of a given sample. A "peak" and "signal" is defined by two values as apparent molecular mass value and an intensity value generated as described. The mass value is an elemental characteristic of a biological entity, whereas the intensity value accords to a certain amount or concentration of a biological entity with the corresponding apparent molecular mass value, and thus "peak" and "signal" always refer to the properties of this biological entity.

The term "cluster" refers to a signal or peak present in a certain set of mass spectra or mass profiles obtained from different samples belonging to two or more different groups (e.g. cancer and non-cancer). Within the said signals belonging to cluster can differ in their intensities, but not in the apparent molecular masses.

The term "variable" refers to a cluster which is subjected to a statistical analysis aiming towards a classification of samples into two or more different sample groups (e.g. cancer and non-cancer) by using decision trees, wherein the sample feature relevant for classification is the intensity value of the variable in the analyzed samples.

Detailed Description of the Invention

a) Principles

The present invention relates to methods for the differential diagnosis of colorectal cancer or a non-malignant disease of the large intestine by detecting one or more differentially expressed biomolecules within a test sample of a given subject, comparing results with samples from healthy subjects, subjects having a precancerous lesion of the large intestine, subjects having colorectal cancer, subjects having a metastasised colorectal cancer, or subjects having a non-malignant disease of the large intestine, wherein the comparison allows for the differential diagnosis of a subject as healthy, having a precancerous lesion of the large intestine, having a colorectal cancer, having a metastasised colorectal cancer or a non-malignant disease of the large intestine.

In one aspect of the invention, a method for the differential diagnosis of a colorectal cancer or a non-malignant disease of the large intestine comprises obtaining a test sample from a given subject, contacting said sample with an adsorbent present on a biologically active surface under specific

binding conditions, allowing the biomolecules within the test sample to bind to said adsorbent, detecting one or more bound biomolecules using a detection method, wherein the detection method generates a mass profile of said sample, transforming mass profile data into a computer-readable form, comparing the mass profile of said sample with a database containing mass profiles from comparable samples specific for healthy subjects, subjects having a precancerous lesion of the large intestine, subjects having a colorectal cancer, subjects having a metastasised colorectal cancer, or subjects having a non-malignant disease of the large intestine. A comparison of mass profiles allows for the medical practitioner to determine if a subject is healthy, has a precancerous lesion of the large intestine, a colorectal cancer, a metastasised colorectal cancer or a non-malignant disease of the large intestine based on the presence, absence or quantity of specific biomolecules.

In more than one embodiment, a single biomolecule or a combination of more than one biomolecule selected from the group having an apparent molecular mass of 2020 Da ± 10 Da, 2049 Da ± 10 Da, 2270 Da ± 11 Da, 2508 Da ± 13 Da, 2732 Da ± 14 Da, 3026 Da ± 15 Da, 3227 Da ± 17 Da, 3326 Da ± 17 Da, 3456 Da ± 17 Da, 3946 Da ± 20 Da, 4103 Da ± 21 Da, 4242 Da ± 21 Da, 4295 Da ± 21 Da, 4359 Da ± 22 Da, 4476 Da ± 22 Da, 4546 Da ± 23 Da, 4697 Da ± 23 Da, 4719 Da ± 24 Da, 4830 Da ± 24 Da, 4965 Da ± 24 Da, 4963 Da ± 25 Da, 5112 Da ± 26 Da, 5225 Da ± 26 Da, 5493 Da ± 27 Da, 5648 Da ± 28 Da, 5772 Da ± 29 Da, 5854 Da ± 29 Da, 6446 Da ± 32 Da, 6644 Da ± 33 Da, 6852 Da ± 34 Da, 6897 Da ± 35 Da, 6999 Da ± 35 Da, 7575 Da ± 38 Da, 7657 Da ± 38 Da, 8076 Da ± 40 Da, 8215 Da ± 41 Da, 8474 Da ± 42 Da, 8574 Da ± 43 Da, 8702 Da ± 44 Da, 8780 Da ± 44 Da, 8922 Da ± 45 Da, 9073 Da ± 45 Da, 9143 Da ± 46 Da, 9201 Da ± 46 Da, 9359 Da ± 47 Da, 9425 Da ± 47 Da, 9581 Da ± 48 Da, 9641 Da ± 48 Da, 9718 Da ± 49 Da, 9930 Da ± 50 Da, 10215 Da ± 51 Da, 10369 Da ± 52 Da, 10440 Da ± 52 Da, 10594 Da ± 53 Da, 11216 Da ± 56 Da, 11464 Da ± 57 Da, 11547 Da ± 58 Da, 11693 Da ± 58 Da, 11905 Da ± 60 Da, 12470 Da ± 62 Da, 12619 Da ± 63 Da, 12828 Da ± 64 Da, 13250 Da ± 66 Da, 13632 Da ± 68 Da, 13784 Da ± 69 Da, 13983 Da ± 70 Da, 14798 Da ± 74 Da, 15005 Da ± 75 Da, 15140 Da ± 76 Da, 15350 Da ± 77 Da, 15879 Da ± 79 Da, 15957 Da ± 80 Da, 16104 Da ± 81 Da, 16164 Da ± 81 Da, 16953 Da ± 85 Da, 17263 Da ± 86 Da, 17397 Da ± 87 Da, 17617 Da ± 88 Da, 17766 Da ± 89 Da, 17890 Da ± 89 Da, 18115 Da ± 91 Da, 18590 Da ± 93 Da, 22338 Da ± 112 Da, 22466 Da ± 112 Da, 22676 Da ± 113 Da, 22951 Da ± 115 Da, 24079 Da ± 120 Da, 28035 Da ± 140 Da, or 28259 Da ± 141 Da may be detected within a given sample. Detection of a single or a combination of more than one biomolecule of the invention is based on specific sample pre-treatment conditions, the pH of binding conditions, and the type of biologically active surface used for the detection of biomolecules. For example, prior to the detection of the biomolecules described herein, a given sample is pre-treated by diluting 1:5 in a denaturation buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, and 2% ampholine. The denatured sample is then diluted 1:10 in a specific binding buffer (0.1 M Tris-HCl, 0.02% Triton X-100, pH 8.5), applied to a biologically active surface comprising of positively-charged quaternary ammonium groups (cationic) and incubated using

specific buffer conditions (0.1 M Tris-HCl, 0.05% Triton X-100, pH 7) to allow for binding of said biomolecules to the above-mentioned biologically active surface.

According to the invention, a biomolecule with the apparent molecular mass of 2020 Da ± 10 Da, 2049 Da ± 10 Da, 2270 Da ± 11 Da, 2308 Da ± 13 Da, 2732 Da ± 14 Da, 3026 Da ± 15 Da, 3227 Da ± 17 Da, 3326 Da ± 17 Da, 3456 Da ± 17 Da, 3546 Da ± 20 Da, 4103 Da ± 21 Da, 4242 Da ± 21 Da, 4295 Da ± 21 Da, 4359 Da ± 22 Da, 4476 Da ± 22 Da, 4546 Da ± 23 Da, 4607 Da ± 23 Da, 4719 Da ± 24 Da, 4830 Da ± 24 Da, 4885 Da ± 24 Da, 4953 Da ± 25 Da, 5112 Da ± 26 Da, 5216 Da ± 26 Da, 5493 Da ± 27 Da, 5648 Da ± 28 Da, 5772 Da ± 29 Da, 5854 Da ± 29 Da, 6446 Da ± 32 Da, 6644 Da ± 33 Da, 6832 Da ± 34 Da, 6897 Da ± 34 Da, 6999 Da ± 35 Da, 7275 Da ± 36 Da, 7637 Da ± 38 Da, 8076 Da ± 40 Da, 8215 Da ± 41 Da, 8474 Da ± 42 Da, 8574 Da ± 43 Da, 8702 Da ± 44 Da, 8780 Da ± 44 Da, 8922 Da ± 45 Da, 9078 Da ± 45 Da, 9143 Da ± 46 Da, 9201 Da ± 46 Da, 9359 Da ± 47 Da, 9425 Da ± 47 Da, 9581 Da ± 48 Da, 9641 Da ± 48 Da, 9718 Da ± 49 Da, 9930 Da ± 50 Da, 10215 Da ± 51 Da, 10369 Da ± 52 Da, 10440 Da ± 52 Da, 10594 Da ± 53 Da, 11216 Da ± 56 Da, 11464 Da ± 57 Da, 11547 Da ± 58 Da, 11693 Da ± 58 Da, 11905 Da ± 60 Da, 12470 Da ± 62 Da, 12619 Da ± 63 Da, 12828 Da ± 64 Da, 13190 Da ± 66 Da, 13452 Da ± 68 Da, 13784 Da ± 69 Da, 13983 Da ± 70 Da, 14798 Da ± 74 Da, 15004 Da ± 75 Da, 15148 Da ± 76 Da, 15350 Da ± 77 Da, 15879 Da ± 79 Da, 15957 Da ± 80 Da, 16104 Da ± 81 Da, 16164 Da ± 81 Da, 16953 Da ± 85 Da, 17263 Da ± 86 Da, 17397 Da ± 87 Da, 17617 Da ± 88 Da, 17766 Da ± 89 Da, 17890 Da ± 89 Da, 18115 Da ± 91 Da, 18390 Da ± 92 Da, 22338 Da ± 112 Da, 22466 Da ± 112 Da, 22676 Da ± 113 Da, 22951 Da ± 115 Da, 24079 Da ± 120 Da, 25095 Da ± 140 Da, or 28250 Da ± 141 Da can be assigned to differentiation between healthy subjects, subjects having a precancerous lesion of the large intestine, subjects having colorectal cancer, subjects having a metastasized colorectal cancer or subjects with a non-malignant disease of the large intestine, and thus are useful as an aid in the diagnosis of a colorectal cancer and/or a non-malignant disease of the large intestine within a subject. Alternatively, said biomolecule may be used to diagnose a subject as healthy.

A biomolecule of the invention may include any molecule that is produced by a cell or living organism, and may have any biochemical property (e.g. phosphorylated protein, positively charged molecules, negatively charged molecules, hydrophobicity, hydrophilicity), but preferably biochemical properties that allow binding of the biomolecule to a biologically active surface comprising positively charged quaternary ammonium groups after incubation in 3 DC mM, 2 M chaotrop, 4% DHAPS, 1% DTE, and 2% Ampholine, and then 1:10 in binding buffer containing 0.1 M Tris-HCl, 0.02% Triton X-100 at pH 9.5 at 0 to 8°C, applying this bound biomolecule to a biologically active surface comprising positively charged (cationic) quaternary ammonium groups (active exchanger), incubating for 120 minutes at 20 to 24°C, and subjecting the bound biomolecule to gas phase ion spectrometry as described in another section.

A biomolecule of the invention may include any molecule that is produced by a cell or living organism, and may have any biochemical property (e.g. phosphorylated protein, positively charged molecules, negatively charged molecules, hydrophobicity, hydrophilicity), but preferably biochemical properties that allow binding of the biomolecule to a biologically active surface comprising positively charged quaternary ammonium groups after incubation in 3 DC mM, 2 M chaotrop, 4% DHAPS, 1% DTE, and 2% Ampholine, and then 1:10 in binding buffer containing 0.1 M Tris-HCl, 0.02% Triton X-100 at pH 9.5 at 0 to 8°C, applying this bound biomolecule to a biologically active surface comprising positively charged (cationic) quaternary ammonium groups (active exchanger), incubating for 120 minutes at 20 to 24°C, and subjecting the bound biomolecule to gas phase ion spectrometry as described in another section.

carbohydrate, lipids and combinations thereof (e.g., glycoproteins, ribonucleoproteins, lipoproteins). Preferably a biomolecule may be a nucleotide, polynucleotide, peptide, protein or fragments thereof. Even more preferred are peptide or protein biomolecules or fragments thereof.

The methods for detecting these biomolecules have many applications. For example, a single biomolecule or a combination of more than one biomolecule selected from the group having an apparent molecular mass of 2020 Da ± 10 Da, 2049 Da ± 10 Da, 2270 Da ± 11 Da, 2308 Da ± 13 Da, 2732 Da ± 14 Da, 3026 Da ± 15 Da, 3227 Da ± 17 Da, 3326 Da ± 17 Da, 3456 Da ± 17 Da, 3546 Da ± 20 Da, 4103 Da ± 21 Da, 4242 Da ± 21 Da, 4295 Da ± 21 Da, 4359 Da ± 22 Da, 4476 Da ± 22 Da, 4546 Da ± 23 Da, 4607 Da ± 23 Da, 4719 Da ± 24 Da, 4830 Da ± 24 Da, 4885 Da ± 24 Da, 4953 Da ± 25 Da, 5112 Da ± 26 Da, 5216 Da ± 26 Da, 5493 Da ± 27 Da, 5648 Da ± 28 Da, 5772 Da ± 29 Da, 5854 Da ± 29 Da, 6446 Da ± 32 Da, 6644 Da ± 33 Da, 6832 Da ± 34 Da, 6897 Da ± 34 Da, 6999 Da ± 35 Da, 7275 Da ± 36 Da, 7637 Da ± 38 Da, 8076 Da ± 40 Da, 8215 Da ± 41 Da, 8474 Da ± 42 Da, 8574 Da ± 43 Da, 8702 Da ± 44 Da, 8780 Da ± 44 Da, 8922 Da ± 45 Da, 9078 Da ± 45 Da, 9143 Da ± 46 Da, 9201 Da ± 46 Da, 9359 Da ± 47 Da, 9425 Da ± 47 Da, 9581 Da ± 48 Da, 9641 Da ± 48 Da, 9718 Da ± 49 Da, 9930 Da ± 50 Da, 10215 Da ± 51 Da, 10369 Da ± 52 Da, 10440 Da ± 52 Da, 10594 Da ± 53 Da, 11216 Da ± 56 Da, 11464 Da ± 57 Da, 11547 Da ± 58 Da, 11693 Da ± 58 Da, 11905 Da ± 60 Da, 12470 Da ± 62 Da, 12619 Da ± 63 Da, 12828 Da ± 64 Da, 13190 Da ± 66 Da, 13452 Da ± 68 Da, 13784 Da ± 69 Da, 13983 Da ± 70 Da, 14798 Da ± 74 Da, 15004 Da ± 75 Da, 15148 Da ± 76 Da, 15350 Da ± 77 Da, 15879 Da ± 79 Da, 15957 Da ± 80 Da, 16104 Da ± 81 Da, 16164 Da ± 81 Da, 16953 Da ± 85 Da, 17263 Da ± 86 Da, 17397 Da ± 87 Da, 17617 Da ± 88 Da, 17766 Da ± 89 Da, 17890 Da ± 89 Da, 18115 Da ± 91 Da, 18390 Da ± 92 Da, 22338 Da ± 112 Da, 22466 Da ± 112 Da, 22676 Da ± 113 Da, 22951 Da ± 115 Da, 24079 Da ± 120 Da, 25095 Da ± 140 Da, or 28250 Da ± 141 Da can be assigned to differentiation between healthy subjects, subjects having a precancerous lesion of the large intestine, subjects having colorectal cancer, subjects having a metastasized colorectal cancer or subjects with a non-malignant disease of the large intestine, and thus are useful as an aid in the diagnosis of a colorectal cancer and/or a non-malignant disease of the large intestine within a subject. Alternatively, said biomolecule may be used to diagnose a subject as healthy.

For example, a biomolecule having the apparent molecular mass of about e.g. 4242 Da is present only in biological samples from patients having a metastasized colorectal cancer. Mass profiling of two test samples from different subjects, X and Y, reveals the presence of a biomolecule with the apparent molecular mass of about 4242 Da in a sample from test subject X, and the absence of said biomolecule in test sample from subject Y. The medical practitioner is able to diagnose subject X as having a metastasized colorectal cancer and subject Y as not having a metastasized colorectal cancer. As yet another example, three biomolecules having the apparent molecular mass of about 5772 Da, 2020 Da and 22951 Da are present in varying quantities in samples specific for precancerous lesions and

"early" colorectal cancer. The biomolecule having the apparent molecular mass of 5772 Da is more present in samples specific for precancerous lesions of the large intestine than for "early" colorectal cancers. A biomolecule having an apparent molecular mass of 2020 Da is detected in samples from subjects having "early" colorectal cancer but not in those having a precancerous lesion, whereas the biomolecule having the molecular mass of 22951 Da is present in about the same quantity in both sample types. Said biomolecules are not present in samples from healthy subjects, only those of apparent molecular mass of 8781 Da and 16104 Da. Analysis of a test sample reveals the presence of biomolecules having the molecular mass of 22951 Da, 5772 Da and 8781 Da. Comparison of the quantity of the biomolecules within said sample reveals that the biomolecule with an apparent molecular mass of 5772 Da is present at lower levels than those found in samples from subjects having a precancerous lesion. The medical practitioner is able to diagnose the test subject as having an "early" colorectal cancer. These examples are solely used for the purpose of clarification and are not intended to limit the scope of this invention.

In another aspect of the invention, an immunoassay can be used to determine the presence or absence of a biomolecule within a test sample of a subject. Thus, the presence or absence of a biomolecule within a sample can be detected using the various immunoassay methods known to those skilled in the art (e.g. ELISA, western blot). If a biomolecule is present in the test sample, it will form an antibody-marker complex with an antibody that specifically binds a biomolecule under suitable incubation conditions. The amount of an antibody-biomolecule complex can be determined by comparing to a standard.

Thus, the invention provides a method for the differential diagnosis of a colorectal cancer and/or a non-malignant tissues of the large intestine comprising detecting of one or more differentially expressed biomolecules within a sample. This method comprises obtaining a test sample from a subject, contacting said sample with a binding moieties specific for a differentially expressed polypeptide, detecting an interaction between the binding moieties and the specific polypeptide, wherein the detection of an interaction indicates the presence or absence of said polypeptide, thereby allowing for the differential diagnosis of a subject as healthy, having a precancerous lesion of the large intestine, having a colorectal cancer, having a malignant colorectal cancer and/or a non-malignant disease of the large intestine. Binding molecules include, but are not limited to, proteins, peptides, antibodies, nucleic acids, hormones, amino acids, sugars, fatty acids, steroids, polynucleotides, carbohydrates, lipids, or a combination thereof (eg. glycoproteins, ribonucleoproteins, lipoproteins), compounds or synthetic molecules. Preferably, binding molecules are antibodies specific for biomolecules selected from the group of having an apparent molecular mass of 2020 Da ± 10 Da, 2049 Da ± 10 Da, 2270 Da ± 11 Da, 2508 Da ± 13 Da, 2732 Da ± 14 Da, 3026 Da ± 15 Da, 3227 Da ± 17 Da, 3326 Da ± 17 Da, 3456 Da ± 17 Da, 3946 Da ± 20 Da, 4103 Da ± 21 Da, 4242 Da ± 21 Da, 4295

Da ± 21 Da, 4359 Da ± 22 Da, 4476 Da ± 22 Da, 4546 Da ± 23 Da, 4607 Da ± 23 Da, 4719 Da ± 24 Da, 4830 Da ± 24 Da, 4865 Da ± 24 Da, 4963 Da ± 25 Da, 5112 Da ± 26 Da, 5226 Da ± 26 Da, 5493 Da ± 27 Da, 5648 Da ± 28 Da, 5772 Da ± 29 Da, 5854 Da ± 29 Da, 6446 Da ± 32 Da, 6644 Da ± 33 Da, 6852 Da ± 34 Da, 6897 Da ± 34 Da, 6999 Da ± 35 Da, 7575 Da ± 38 Da, 7657 Da ± 38 Da, 8076 Da ± 40 Da, 8215 Da ± 41 Da, 8474 Da ± 42 Da, 8574 Da ± 43 Da, 8702 Da ± 44 Da, 8780 Da ± 44 Da, 8922 Da ± 45 Da, 9078 Da ± 45 Da, 9143 Da ± 46 Da, 9201 Da ± 46 Da, 9359 Da ± 47 Da, 9425 Da ± 47 Da, 9581 Da ± 48 Da, 9641 Da ± 48 Da, 9718 Da ± 49 Da, 9930 Da ± 50 Da, 10215 Da ± 51 Da, 10369 Da ± 52 Da, 10440 Da ± 52 Da, 10594 Da ± 53 Da, 11216 Da ± 56 Da, 11464 Da ± 57 Da, 11547 Da ± 58 Da, 11693 Da ± 58 Da, 11905 Da ± 60 Da, 12470 Da ± 62 Da, 12619 Da ± 63 Da, 12828 Da ± 64 Da, 13290 Da ± 66 Da, 13632 Da ± 68 Da, 13784 Da ± 69 Da, 13983 Da ± 70 Da, 14798 Da ± 74 Da, 15005 Da ± 75 Da, 15140 Da ± 76 Da, 15350 Da ± 77 Da, 15879 Da ± 79 Da, 15957 Da ± 80 Da, 16104 Da ± 81 Da, 16164 Da ± 81 Da, 16933 Da ± 85 Da, 17263 Da ± 86 Da, 17397 Da ± 87 Da, 17617 Da ± 88 Da, 17766 Da ± 89 Da, 17890 Da ± 89 Da, 18115 Da ± 91 Da, 18390 Da ± 92 Da, 22338 Da ± 112 Da, 22466 Da ± 112 Da, 22676 Da ± 113 Da, 22951 Da ± 115 Da, 24079 Da ± 120 Da, 28055 Da ± 140 Da, or 28259 Da ± 141 Da

In another aspect of the invention, a method for detecting the differential presence of one or more biomolecules selected from the group having an apparent molecular mass of 2020 Da ± 10 Da, 2049 Da ± 10 Da, 2270 Da ± 11 Da, 2508 Da ± 13 Da, 2732 Da ± 14 Da, 3026 Da ± 15 Da, 3227 Da ± 17 Da, 3326 Da ± 17 Da, 3456 Da ± 17 Da, 3946 Da ± 20 Da, 4103 Da ± 21 Da, 4242 Da ± 21 Da, 4295 Da ± 21 Da, 4359 Da ± 22 Da, 4476 Da ± 22 Da, 4546 Da ± 23 Da, 4607 Da ± 23 Da, 4719 Da ± 24 Da, 4830 Da ± 24 Da, 4865 Da ± 24 Da, 4963 Da ± 25 Da, 5112 Da ± 26 Da, 5226 Da ± 26 Da, 5493 Da ± 27 Da, 5648 Da ± 28 Da, 5772 Da ± 29 Da, 5854 Da ± 29 Da, 6446 Da ± 32 Da, 6644 Da ± 33 Da, 6852 Da ± 34 Da, 6897 Da ± 34 Da, 6999 Da ± 35 Da, 7575 Da ± 38 Da, 7657 Da ± 38 Da, 8076 Da ± 40 Da, 8215 Da ± 41 Da, 8474 Da ± 42 Da, 8574 Da ± 43 Da, 8702 Da ± 44 Da, 8780 Da ± 44 Da, 8922 Da ± 45 Da, 9078 Da ± 45 Da, 9143 Da ± 46 Da, 9201 Da ± 46 Da, 9359 Da ± 47 Da, 9425 Da ± 47 Da, 9581 Da ± 48 Da, 9641 Da ± 48 Da, 9718 Da ± 49 Da, 9930 Da ± 50 Da, 10215 Da ± 51 Da, 10369 Da ± 52 Da, 10440 Da ± 52 Da, 10594 Da ± 53 Da, 11216 Da ± 56 Da, 11464 Da ± 57 Da, 11547 Da ± 58 Da, 11693 Da ± 58 Da, 11905 Da ± 60 Da, 12470 Da ± 62 Da, 12619 Da ± 63 Da, 12828 Da ± 64 Da, 13290 Da ± 66 Da, 13632 Da ± 68 Da, 13784 Da ± 69 Da, 13983 Da ± 70 Da, 14798 Da ± 74 Da, 15005 Da ± 75 Da, 15140 Da ± 76 Da, 15350 Da ± 77 Da, 15879 Da ± 79 Da, 15957 Da ± 80 Da, 16104 Da ± 81 Da, 16164 Da ± 81 Da, 16933 Da ± 85 Da, 17263 Da ± 86 Da, 17397 Da ± 87 Da, 17617 Da ± 88 Da, 17766 Da ± 89 Da, 17890 Da ± 89 Da, 18115 Da ± 91 Da, 18390 Da ± 92 Da, 22338 Da ± 112 Da, 22466 Da ± 112 Da, 22676 Da ± 113 Da, 22951 Da ± 115 Da, 24079 Da ± 120 Da, 28055 Da ± 140 Da, or 28259 Da ± 141 Da in a test sample of a subject involves contacting the test sample with a compound or agent capable of detecting said biomolecule such that the presence of said biomolecule is directly and/or indirectly labelled. For example a fluorescently

labelled secondary antibody can be used to detect a primary antibody bound to its specific biomolecule. Furthermore, such detection methods can be used to detect a variety of biomolecules within a test sample both *in vitro* as well as *in vivo*.

For example, *in vivo*, antibodies or fragments thereof may be utilised for the detection of a biomolecule in a biological sample comprising: applying a labelled antibody directed against a given biomolecule of the invention to said sample under conditions that favour an interaction between the labelled antibody and its corresponding protein. Depending on the nature of the biological sample, it is possible to determine not only the presence of a biomolecule, but also its cellular distribution. For example, in a blood serum sample, only the serum levels of a given biomolecule can be detected, whereas its level of expression and cellular localisation can be detected in histological samples. It will be obvious to those skilled in the art, that a wide variety of methods can be modified in order to achieve such detection.

For example, an antibody coupled to an enzyme is detected using a chromogenic substrate that is recognised and cleaved by the enzyme to produce a chemical moiety, which is readily detected using spectrometric, fluorimetric or visual means. Enzymes used to for labelling include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. Detection may also be accomplished by visual comparison of the extent of the enzymatic reaction of a substrate with that of similarly prepared standards. Alternatively, radiolabelled antibodies can be detected using a gamma or a scintillation counter, or they can be detected using autoradiography. In another example, fluorescently labelled antibodies are detected based on the level at which the attached compound fluoresces following exposure to a given wavelength. Fluorescent compounds typically used in antibody labelling include, but are not limited to, fluorescein isothiocynate, rhodamine, phycoerthyrin, phycocyanin, allophycocyani, o-phthaldehyde and fluorescamine. In yet another example, antibodies coupled to a chemi- or bioluminescent compound can be detected by determining the presence of luminescence. Such compounds include, but are not limited to, luminal, isoluminal, theromatic acridinium ester, imidazole, acridinium salt, oxalate ester, luciferin, luciferase and aequorin.

Furthermore, *in vivo* techniques for the detection of a biomolecule of the invention include introducing into a subject a labelled antibody directed against a given polypeptide or fragment thereof.

In more than one embodiment of the invention, the test sample used for the differential diagnosis of a colorectal cancer and/or a non-malignant disease of the large intestine of a subject may be of blood, blood serum, plasma, nipple aspirate, urine, semen, seminal fluid, seminal plasma, prostatic fluid, excreta, tears, saliva, sweat, biopsy, ascites, cerebrospinal fluid, milk, lymph, or tissue extract origin. Preferably, test samples are of blood, blood serum, plasma, urine, excreta, prostatic fluid, biopsy, ascites, lymph or tissue extract origin. More preferred are blood, blood serum, plasma, urine, excreta, biopsy, lymph or tissue extract samples. Even more preferred are blood serum, urine, excreta or biopsy samples. Overall preferred are blood serum samples.

Furthermore, test samples used for the methods of the invention are isolated from subjects of mammalian origin, preferably of primate origin. Even more preferred are subjects of human origin.

In addition, the methods of the invention for the differential diagnosis of healthy subjects, subjects having a precancerous lesion of the large intestine, subjects having a colorectal cancer, subjects having a metastasized colorectal cancer or subjects having a non-malignant disease of the large intestine described herein may be combined with other diagnostic methods to improve the outcome of the differential diagnosis. Other diagnostic methods are known to those skilled in the art.

b) Database

In another aspect of the invention, a database comprising of mass profiles specific for healthy subjects, subjects having a precancerous lesion of the large intestine, subjects having a colorectal cancer, subjects having a metastasised colorectal cancer, or subjects having a non-malignant disease of the large intestine is generated by contacting biological samples isolated from above-mentioned subjects with an adsorbent on a biologically active surface under specific binding conditions, allowing the biomolecules within said sample to bind said adsorbent, detecting one or more bound biomolecules using a detection method wherein the detection method generates a mass profile of said sample, transforming the mass profile data into a computer-readable form and applying a mathematical algorithm to classify the mass profile as specific for healthy subjects, subjects having a precancerous lesion of the large intestine, subjects having a colorectal cancer, subjects having a metastasised colorectal cancer, or subjects having a non-malignant disease of the large intestine.

According to the invention, the classification of said mass profiles is performed using the "CART" decision tree approach (classification and regression trees; Breiman et al., 1984) and is known to those skilled in the art. Furthermore, bagging of classifiers is applied to overcome typical instabilities of forward variable selection procedures, thereby increasing overall classifier performance (Breiman, 1994).

In more than one embodiment, one or more biomolecules selected from the group having an apparent molecular mass of 2020 Da ± 10 Da, 2049 Da ± 10 Da, 2270 Da ± 11 Da, 2508 Da ± 13 Da, 2732 Da ± 14 Da, 3026 Da ± 15 Da, 3227 Da ± 17 Da, 3326 Da ± 17 Da, 3456 Da ± 17 Da, 3946 Da ± 20 Da, 4103 Da ± 21 Da, 4242 Da ± 21 Da, 4295 Da ± 21 Da, 4359 Da ± 22 Da, 4476 Da ± 22 Da, 4546 Da ± 23 Da, 4607 Da ± 23 Da, 4719 Da ± 24 Da, 4830 Da ± 24 Da, 4865 Da ± 24 Da, 4963 Da ± 25 Da, 5112 Da ± 26 Da, 5226 Da ± 26 Da, 5493 Da ± 27 Da, 5648 Da ± 28 Da, 5772 Da ± 29 Da, 5854 Da ± 29 Da, 6446 Da ± 32 Da, 6644 Da ± 33 Da, 6852 Da ± 34 Da, 6897 Da ± 34 Da, 6999 Da ± 35 Da, 7575 Da ± 38 Da, 7657 Da ± 38 Da, 8076 Da ± 40 Da, 8215 Da ± 41 Da, 8474 Da ± 42 Da, 8574 Da ± 43 Da, 8702 Da ± 44 Da, 8780 Da ± 44 Da, 8922 Da ± 45 Da, 9078 Da ± 45 Da, 9143 Da ± 46 Da, 9201 Da ± 46 Da, 9359 Da ± 47 Da, 9425 Da ± 47 Da, 9581 Da ± 48 Da, 9641 Da ± 48 Da, 9718 Da ± 49 Da, 9930 Da ± 50 Da, 10215 Da ± 51 Da, 10369 Da ± 52 Da, 10440 Da ± 52 Da, 10594 Da ± 53 Da, 11216 Da ± 56 Da, 11464 Da ± 57 Da, 11547 Da ± 58 Da, 11693 Da ± 58 Da, 11905 Da ± 60 Da, 12470 Da ± 62 Da, 12619 Da ± 63 Da, 12828 Da ± 64 Da, 13290 Da ± 66 Da, 13632 Da ± 68 Da, 13784 Da ± 69 Da, 13983 Da ± 70 Da, 14798 Da ± 74 Da, 15005 Da ± 75 Da, 15140 Da ± 76 Da, 15350 Da ± 77 Da, 15879 Da ± 79 Da, 15957 Da ± 80 Da, 16104 Da ± 81 Da, 16164 Da ± 81 Da, 16953 Da ± 85 Da, 17263 Da ± 86 Da, 17397 Da ± 87 Da, 17617 Da ± 88 Da, 17766 Da ± 89 Da, 17890 Da ± 89 Da, 18115 Da ± 91 Da, 18390 Da ± 92 Da, 22338 Da ± 112 Da, 22466 Da ± 112 Da, 22676 Da ± 113 Da, 22951 Da ± 115 Da, 24079 Da ± 120 Da, 28055 Da ± 140 Da, or 28259 Da ± 141 Da may be detected within a given biological sample. Detection of said biomolecules of the invention is based on specific sample pre-treatment conditions, the pH of binding conditions, and the type of biologically active surface used for the detection of biomolecules.

Within the context of the invention, biomolecules within a given sample are bound to an adsorbent on a biologically active surface under specific binding conditions, for example, the biomolecules within a given sample are applied to a biologically active surface comprising positively-charged quaternary ammonium groups (cationic) and incubated with 0.1 M Tris-HCl, 0.02% Triton X-100 at a pH of 8.5 to allow for specific binding. Biomolecules that bind to said biologically active surface under these conditions are negatively charged molecules. It should be noted that although the biomolecules of the invention are bound to a cationic adsorbent comprising of positively-charged quaternary ammonium groups, the biomolecules are capable of binding other types of adsorbents, as described in another section using binding conditions known to those skilled in the art. Accordingly, some embodiments of the invention are not limited to the use of cationic adsorbents.

According to the invention, a biomolecule with the molecular mass of 2020 Da ± 10 Da, 2049 Da ± 10 Da, 2270 Da ± 11 Da, 2508 Da ± 13 Da, 2732 Da ± 14 Da, 3026 Da ± 15 Da, 3227 Da ± 17 Da, 3326 Da ± 17 Da, 3456 Da ± 17 Da, 3946 Da ± 20 Da, 4103 Da ± 21 Da, 4242 Da ± 21 Da, 4295 Da ± 21 Da, 4359 Da ± 22 Da, 4476 Da ± 22 Da, 4546 Da ± 23 Da, 4607 Da ± 23 Da, 4719 Da ± 24 Da, 4830

Da ± 24 Da, 4865 Da ± 24 Da, 4963 Da ± 25 Da, 5112 Da ± 26 Da, 5226 Da ± 26 Da, 5493 Da ± 27 Da, 5648 Da ± 28 Da, 5772 Da ± 29 Da, 5854 Da ± 29 Da, 6446 Da ± 32 Da, 6644 Da ± 33 Da, 6852 Da ± 34 Da, 6897 Da ± 34 Da, 6999 Da ± 35 Da, 7575 Da ± 38 Da, 7657 Da ± 38 Da, 8076 Da ± 40 Da, 8215 Da ± 41 Da, 8474 Da ± 42 Da, 8574 Da ± 43 Da, 8702 Da ± 44 Da, 8780 Da ± 44 Da, 8922 Da ± 45 Da, 9078 Da ± 45 Da, 9143 Da ± 46 Da, 9201 Da ± 46 Da, 9359 Da ± 47 Da, 9425 Da ± 47 Da, 9581 Da ± 48 Da, 9641 Da ± 48 Da, 9718 Da ± 49 Da, 9930 Da ± 50 Da, 10215 Da ± 51 Da, 10369 Da ± 52 Da, 10440 Da ± 52 Da, 10594 Da ± 53 Da, 11216 Da ± 56 Da, 11464 Da ± 57 Da, 11547 Da ± 58 Da, 11693 Da ± 58 Da, 11905 Da ± 60 Da, 12470 Da ± 62 Da, 12619 Da ± 63 Da, 12828 Da ± 64 Da, 13290 Da ± 66 Da, 13632 Da ± 68 Da, 13784 Da ± 69 Da, 13983 Da ± 70 Da, 14798 Da ± 74 Da, 15005 Da ± 75 Da, 15140 Da ± 76 Da, 15350 Da ± 77 Da, 15879 Da ± 79 Da, 15957 Da ± 80 Da, 16104 Da ± 81 Da, 16164 Da ± 81 Da, 16953 Da ± 85 Da, 17263 Da ± 86 Da, 17397 Da ± 87 Da, 17617 Da ± 88 Da, 17766 Da ± 89 Da, 17890 Da ± 89 Da, 18115 Da ± 91 Da, 18390 Da ± 92 Da, 22338 Da ± 112 Da, 22466 Da ± 112 Da, 22676 Da ± 113 Da, 22951 Da ± 115 Da, 24079 Da ± 120 Da, 28055 Da ± 140 Da, or 28259 Da ± 141 Da is detected by diluting the biological sample 1:5 in a denaturation buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, and 2% Ampholine, and then 1:10 in binding buffer consisting of 0.1 M Tris-HCl, 0.02% Triton X-100 at pH 8.5 at 0 to 4°C, applying thus treated sample to a biologically active surface comprising positively charged (cationic) quaternary ammonium groups (anion exchanging), incubating for 120 minutes at 20 to 24°C, and subjecting the bound biomolecules to gas phase ion spectrometry as described in another section.

In one embodiment of the invention, biological samples used to generate a database of mass profiles for healthy subjects, subjects having a precancerous lesion of the large intestine, subjects having a colorectal cancer, subjects having a metastasised colorectal cancer or subjects having a non-malignant disease of the large intestine, may be of blood, blood serum, plasma, nipple aspirate, urine, semen, seminal fluid, seminal plasma, prostatic fluid, excreta, tears, saliva, sweat, biopsy, ascites, cerebrospinal fluid, milk, lymph, or tissue extract origin. Preferably, biological samples are of blood, blood serum, plasma, urine, excreta, prostatic fluid, biopsy, ascites, lymph or tissue extract origin. More preferred are blood, blood serum, plasma, urine, excreta, biopsy, lymph or tissue extract samples. Even more preferred are blood serum, urine, excreta or biopsy samples. Overall preferred are blood serum samples.

Furthermore, the biological samples related to the invention are isolated from subjects considered to be healthy, having a precancerous lesion of the large intestine, having a colorectal cancer, having a metastasised colorectal cancer or having a non-malignant disease of the large intestine. Said subjects are of mammalian origin, preferably of primate origin. Even more preferred are subjects of human origin.

A subject of the invention that is said to have a precancerous lesion of the large intestine, displays preliminary stages of a cancer (i.e. dysplasia), wherein a cell and/or tissue has become susceptible to the development of a cancer as a result of either a genetic predisposition, exposure to a cancer-causing agent (carcinogen) or both.

A genetic pre-disposition may include a predisposition for an autosomal dominant inherited cancer syndrome which is generally indicated by a strong family history of uncommon cancer and/or an association with a specific marker phenotype (e.g. familial adenomatous polyps of the colon), a familial cancer wherein an evident clustering of cancer is observed but the role of inherited predisposition may not be clear (e.g. breast cancer, ovarian cancer, or colon cancer), or an autosomal recessive syndrome characterised by chromosomal or DNA instability. Whereas, cancer-causing agents include agents that cause genetic damage and induce neoplastic transformation of a cell. Such agents fall into three categories: 1) chemical carcinogens such as alkylating agents, polycyclic aromatic hydrocarbons, aromatic amines, azo dyes, nitrosamines and amides, asbestos, vinyl chloride, chromium, nickel, arsenic, and naturally occurring carcinogens (e.g. aflotoxin B1); 2) radiation such as ultraviolet (UV) and ionisation radiation including electromagnetic (e.g. x-rays, γ-rays) and particulate radiation (e.g. α and β particles, protons, neutrons); 3) viral and microbial carcinogens such as human Papillomavirus (HPV), Epstein-Barr virus (EBV), hepatitis B virus (HBV), human T-cell leukaemia virus type I (HTLV-1), or *Helicobacter pylori*.

Alternatively, a subject within the invention that is said to have a colorectal cancer possesses a cancer that arises from the large intestine (interchangeably referred to as colorectal cancers within the invention). Such cancers may include, but are not limited to, colon and rectal cancers.

Within the context of the invention, cancers of large intestine (interchangably referred to as colorectal cancers within the invention) may also be of various stages, wherein the staging is based on the size of the primary lesion, its extent of spread to regional lymph nodes, and the presence or absence of blood-borne metastases (metastatic colorectal cancers. The various stages of a cancer may be identified using staging systems known to those skilled in the art [e.g. Union Internationale Contre Cancer (UICC) system or American Joint Committee on Cancer (AJC)]. Also included are different grades of said cancers, wherein the grade of a cancer is based on the degree of differentiation of the epithelial cells within the lining of the large intestine and the number of mitoses as a correlation to a neoplasm's aggression.

Healthy individuals, as related to certain embodiments of the invention, are those that possess good health, and demonstrate an absence of a colorectal cancer or a non-malignant disease of the large

intestine.

e) Biomolecules

The differential expression of biomolecules in samples from healthy subjects, subjects having a precancerous lesion of the large intestine, subjects having a colorectal cancer, subjects having metastasised colorectal cancer, and subjects having a non-malignant disease of the large intestine, allows for the differential diagnosis of a non-malignant disease or a cancer of the large intestine wihin a subject.

Biomolecules are said to be specific for a particular clinical state (e.g. healthy, precancerous lesion of the large intestine, colorectal cancer, metastasised colorectal cancer, a non-malignant disease of the large intestine) when they are present at different levels within samples taken from subjects in one clinical state as compared to samples taken from subjects from other clinical states (e.g. in subjects with a precancerous lesion of the large intestine vs. in subjects with a metastasised colorectal cancer). Biomolecules may be present at elevated levels, at decreased levels, or altogether absent within a sample taken from a subject in a particular clinical state (e.g. healthy, precancerous lesion of the large intestine, colorectal cancer, metastasised colorectal cancer, a non-malignant disease of the large intestine). For example, biomolecules A and B are found at elevated levels in samples isolated from healthy subjects as compared to samples isolated from subjects having a precancerous lesion of the large intestine, a colorectal cancer, a metastatic colorectal cancer or a non-malignant disease of the large intestine. Whereas, biomolecules X, Y, Z are found at elevated levels and/or more frequently in samples isolated from subjects having a precancerous lesion of the large intestine as opposed to subjects in good health, having a colorectal cancer, a metastasised colorectal cancer or a non-malignant disease of the large intestine. Biomolecules A and B are said to be specific for healthy subjects, whereas biomolecules X, Y, Z are specific for subjects having a precancerous lesion of the large intestine.

Accordingly, the differential presence of one or more biomolecules found in a test sample compared to samples from healthy subjects, subjects with a precancerous lesion of the large intestine, a colorectal cancer, a metastasized colorectal cancer, or a non-malignant disease of the large intestine, or the mere detection of one or more biomolecules in the test sample provides useful information regarding probability of whether a subject being tested has a precancerous lesion of the large intestine, a colorectal cancer, a metastasized colorectal cancer or a non-malignant disease of the large intestine. The probability that a subject being tested has a precancerous lesion of the large intestine, a colorectal cancer, a metastasized colorectal cancer or a non-malignant disease of the large intestine depends on whether the quantity of one or more biomolecules in a test sample taken from said subject is statistically significantly different from the quantity of one or more biomolecules in a biological

sample taken from healthy subjects, subjects having a precancerous lesion of the large intestine, a colorectal cancer, a metastasized colorectal cancer, or a non-malignant disease of the large intestine.

A biomolecule of the invention may be any molecule that is produced by a cell or living organism, and may have any biochemical property (e.g. phosphorylated protein, positively charged molecules, negatively charged molecules, hydrophobicity, hydrophilicity), but preferably biochemical properties that allow binding of the biomolecule to a biologically active surface comprising positively charged quaternary ammonium groups after denaturation in 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, and 2% Ampholine and dilution in 0.1 M Tris-HCl, 0.02% Triton X-100 at pH 8.5 at 0 to 4°C followed by incubation on said biologically active surface for 120 minutes at 20 to 24°C. Such molecules include, but are not limited to, molecules comprising nucleotides, amino acids, sugars, fatty acids, steroids, nucleic acids, polynucleotides (DNA or RNA), polypeptides, proteins, antibodies, carbohydrates, lipids, and combinations thereof (e.g., glycoproteins, ribonucleoproteins, lipoproteins). Preferably a biomolecule may be a nucleotide, polynucleotide, peptide, protein, or fragments thereof. Even more preferred are peptide or protein biomolecules.

The biomolecules of the invention can be detected based on specific sample pre-treatment conditions, the set of binding conditions, the type of biologically active surface used for the detection of biomolecules within a given sample and their molecular mass. For example, prior to the detection of the biomolecules described herein, a given sample is pre-treated by diluting it 1:5 in a denaturation buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, and 2% ampholine. The denatured sample is then diluted 1:10 in 0.1 M Tris-HCl, 0.02% Triton X-100, pH 8.5, applied to a biologically active surface comprising positively charged quaternary ammonium groups (cationic) and incubated using specific buffer conditions (0.1 M Tris-HCl, 0.02% Triton X-100, pH 8.5) to allow for binding of said biomolecules to the above mentioned biologically active surface. It should be noted that although the biomolecules of the invention are detected using a cationic adsorbent positively charged quaternary ammonium groups, as well as specific pre-treatment and binding conditions, the biomolecules are capable of binding other types of adsorbents as described below, using alternative pre-treatment and binding conditions known to those skilled in the art. Accordingly, some embodiments of the invention are not limited to the use of cationic adsorbents.

The biomolecules of the invention include biomolecules having a molecular mass selected from the group consisting of 2020 Da ± 10 Da, 2048 Da ± 10 Da, 2270 Da ± 11 Da, 2508 Da ± 13 Da, 2732 Da ± 14 Da, 3026 Da ± 15 Da, 3227 Da ± 17 Da, 3325 Da ± 17 Da, 3456 Da ± 17 Da, 3946 Da ± 20 Da, 4103 Da ± 21 Da, 4242 Da ± 21 Da, 4295 Da ± 21 Da, 4359 Da ± 22 Da, 4476 Da ± 22 Da, 4546 Da ± 23 Da, 4607 Da ± 23 Da, 4719 Da ± 24 Da, 4830 Da ± 24 Da, 4865 Da ± 24 Da, 4963 Da ± 25 Da, 5112 Da ± 26 Da, 5226 Da ± 26 Da, 5493 Da ± 27 Da, 5648 Da ± 28 Da, 5772 Da ± 29 Da, 5854 Da ± 29 Da, 6446 Da ± 32 Da, 6644 Da ± 33 Da, 6852 Da ± 34 Da, 6897 Da ± 34 Da, 6999 Da ± 35 Da, 7575 Da ± 38 Da, 7657 Da ± 38 Da, 8076 Da ± 40 Da, 8215 Da ± 41 Da, 8474 Da ± 42 Da, 8574 Da ± 43 Da, 8702 Da ± 44 Da, 8780 Da ± 44 Da, 8922 Da ± 45 Da, 9078 Da ± 45 Da, 9143 Da ± 46 Da, 9201 Da ± 46 Da, 9359 Da ± 47 Da, 9425 Da ± 47 Da, 9581 Da ± 48 Da, 9641 Da ± 48 Da, 9718 Da ± 49 Da, 9930 Da ± 50 Da, 10215 Da ± 51 Da, 10369 Da ± 52 Da, 10440 Da ± 52 Da, 10594 Da ± 53 Da, 11216 Da ± 56 Da, 11464 Da ± 57 Da, 11547 Da ± 58 Da, 11693 Da ± 58 Da, 11905 Da ± 60 Da, 12470 Da ± 62 Da, 12619 Da ± 63 Da, 12828 Da ± 64 Da, 13290 Da ± 66 Da, 13632 Da ± 68 Da, 13784 Da ± 69 Da, 13983 Da ± 70 Da, 14798 Da ± 74 Da, 15005 Da ± 75 Da, 15140 Da ± 76 Da, 15350 Da ± 77 Da, 15879 Da ± 79 Da, 15957 Da ± 80 Da, 16104 Da ± 81 Da, 16164 Da ± 81 Da, 16953 Da ± 85 Da, 17263 Da ± 86 Da, 17397 Da ± 87 Da, 17617 Da ± 88 Da, 17766 Da ± 89 Da, 17890 Da ± 89 Da, 18115 Da ± 91 Da, 18390 Da ± 92 Da, 22338 Da ± 112 Da, 22466 Da ± 112 Da, 22676 Da ± 113 Da, 22951 Da ± 115 Da, 24079 Da ± 120 Da, 28055 Da ± 140 Da, or 28259 Da ± 141 Da.

According to the invention, a biomolecule with the molecular mass of 2020 Da ± 10 Da, 2048 Da ± 10 Da, 2270 Da ± 11 Da, 2508 Da ± 13 Da, 2732 Da ± 14 Da, 3026 Da ± 15 Da, 3227 Da ± 17 Da, 3325 Da ± 17 Da, 3456 Da ± 17 Da, 3946 Da ± 20 Da, 4103 Da ± 21 Da, 4242 Da ± 21 Da, 4295 Da ± 21 Da, 4359 Da ± 22 Da, 4476 Da ± 22 Da, 4546 Da ± 23 Da, 4607 Da ± 23 Da, 4719 Da ± 24 Da, 4830 Da ± 24 Da, 4865 Da ± 24 Da, 4963 Da ± 25 Da, 5112 Da ± 26 Da, 5226 Da ± 26 Da, 5493 Da ± 27 Da, 5648 Da ± 28 Da, 5772 Da ± 29 Da, 5854 Da ± 29 Da, 6446 Da ± 32 Da, 6644 Da ± 33 Da, 6852 Da ± 34 Da, 6897 Da ± 34 Da, 6999 Da ± 35 Da, 7575 Da ± 38 Da, 7657 Da ± 38 Da, 8076 Da ± 40 Da, 8215 Da ± 41 Da, 8474 Da ± 42 Da, 8574 Da ± 43 Da, 8702 Da ± 44 Da, 8780 Da ± 44 Da, 8922 Da ± 45 Da, 9078 Da ± 45 Da, 9143 Da ± 46 Da, 9201 Da ± 46 Da, 9359 Da ± 47 Da, 9425 Da ± 47 Da, 9581 Da ± 48 Da, 9641 Da ± 48 Da, 9718 Da ± 49 Da, 9930 Da ± 50 Da, 10215 Da ± 51 Da, 10369 Da ± 52 Da, 10440 Da ± 52 Da, 10594 Da ± 53 Da, 11216 Da ± 56 Da, 11464 Da ± 57 Da, 11547 Da ± 58 Da, 11693 Da ± 58 Da, 11905 Da ± 60 Da, 12470 Da ± 62 Da, 12619 Da ± 63 Da, 12828 Da ± 64 Da, 13290 Da ± 66 Da, 13632 Da ± 68 Da, 13784 Da ± 69 Da, 13983 Da ± 70 Da, 14798 Da ± 74 Da, 15005 Da ± 75 Da, 15140 Da ± 76 Da, 15350 Da ± 77 Da, 15879 Da ± 79 Da, 15957 Da ± 80 Da, 16104 Da ± 81 Da, 16164 Da ± 81 Da, 16953 Da ± 85 Da, 17263 Da ± 86 Da, 17397 Da ± 87 Da, 17617 Da ± 88 Da, 17766 Da ± 89 Da, 17890 Da ± 89 Da, 18115 Da ± 91 Da, 18390 Da ± 92 Da, 22338 Da ± 112 Da, 22466 Da ± 112 Da, 22676 Da ± 113 Da, 22951 Da ± 115 Da, 24079 Da ± 120 Da, 28055 Da ± 140 Da, or 28259 Da ± 141 Da is detected by diluting the biological sample 1:5 in a denaturation buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, and 2% Ampholine, and then 1:10 in binding buffer consisting of 0.1 M Tris-HCl, 0.02% Triton X-100 at pH 8.5 at 0 to 4°C, applying thus treated sample to a biologically active surface comprising positively charged (cationic) quaternary ammonium groups (anion exchanging), incubating for 120 minutes at 20 to 24°C, and subjecting the bound biomolecules to gas phase ion spectrometry as described in another

section.

Although said biomolecules were first identified in blood serum samples, their detection is not limited to said sample type. The biomolecules may also be detected in other samples types, such as blood, blood serum, plasma, nipple aspirate, urine, semen, seminal fluid, seminal plasma, prostatic fluid, excreta, tears, saliva, sweat, biopsy, ascites, cerebrospinal fluid, milk, lymph, or tissue extract. Preferably, samples are of blood, blood serum, plasma, urine, excreta, prostatic fluid, biopsy, ascites, lymph or tissue extract origin. More preferred are blood, blood serum, plasma, urine, excreta, biopsy, lymph, or tissue extract samples. Even more preferred are blood serum, urine, excreta or biopsy samples. Overall preferred are blood serum samples.

Since the biomolecules can be sufficiently characterized by their mass and biochemical characteristics such as the type of biologically active surface they bind to or the pH of binding conditions, it is not necessary to identify the biomolecules in order to be able to identify them in a sample. It should be noted that molecular mass and binding properties are characteristic properties of these biomolecules and not limitations on the means of detection or isolation. Furthermore, using the methods described herein or other methods known in the art, the absolute identity of the markers can be determined. This is important when one wishes to develop and/or screen for specific binding molecules, or to develop an assay for the detection of said biomolecules using specific binding molecules.

d) Biologically Active Surfaces

In one embodiment of the invention, biologically active surfaces include, but are not restricted to, surfaces that contain adsorbents with anion exchange/quaternary ammonium groups (anion exchange surfaces), carboxylate groups (cation exchange surfaces), alkyl or aryl chains (hydrophobic interaction, reverse phase chemistry), groups such as nitrilotriacetic acid that immobilize metal ions such as nickel, gallium, copper, or zinc (metal affinity interaction), or biomolecules such as proteins, preferably antibodies, or nucleic acids, preferably protein binding sequences, covalently bound to the surface via carbonyl diimidazole moieties or epoxy groups (specific affinity interaction). Preferred are adsorbents comprising anion exchange surfaces.

These surfaces may be located on matrices like polysaccharides such as sepharose, e.g. anion exchange surfaces or hydrophobic interaction surfaces, or solid metals, e.g. antibodies coupled to magnetic beads. Surfaces may also include gold-plated surfaces such as those used for Biacore Sensor Chip technology. Other surfaces known to those skilled in the art are also included within the scope of the invention.

Biologically active surfaces are able to adsorb biomolecules like amino acids, sugars, fatty acids,

steroids, nucleic acids, polynucleotides, polypeptides, carbohydrates, lipids, and combinations thereof (e.g., glycoproteins, ribonucleoproteins, lipoproteins).

In another embodiment, devices that use biologically active surfaces to selectively adsorb biomolecules may be chromatography columns for Fast Protein Liquid Chromatography (FPLC) and High Pressure Liquid Chromatography (HPLC), where the matrix, e.g. a polysaccharide, carrying the biologically active surface, is filled into vessels (usually referred to as "columns") made of glass, steel, or synthetic materials like polyetheretherketone (PEEK).

In yet another embodiment, devices that use biologically active surfaces to selectively adsorb biomolecules may be metal strips carrying thin layers of the biologically active surface on one or more spots of the strip surface to be used as probes for gas phase ion spectrometry analysis, for example the SAX2 ProteinChip array (Ciphergen Biosystems, Inc.) for SELDI analysis.

e) Mass Profiling

In one embodiment, the mass profile of a sample may be generated using an array-based assay in which the biomolecules of a given sample are bound by biochemical or affinity interactions to an adsorbent present on a biologically active surface located on a solid platform ("array" or "probe"). After the biomolecules have bound to the adsorbent, they are detected using gas phase ion spectrometry. Biomolecules or other substances bound to the adsorbents on the probes can be analyzed using a gas phase ion spectrometer. This includes, e.g., mass spectrometers, ion mobility spectrometers, or total ion current measuring devices. The quantity and characteristics of the biomolecule can be determined using gas phase ion spectrometry. Other substances in addition to the biomolecule of interest can also be detected by gas phase ion spectrometry.

In one embodiment, a mass spectrometer can be used to detect biomolecules on the probe. In a typical mass spectrometer, a probe with a biomolecule is introduced into an inlet system of the mass spectrometer. The biomolecule is then ionized by an ionization source, such as a laser, fast atom bombardment, or plasma. The generated ions are collected by an ion optic assembly, and then a mass analyzer disperses and analyzes the passing ions. Within the scope of this invention, the ionization source that ionizes the biomolecule is a laser.

The ions exiting the mass analyzer are detected by a ion detector. The ion detector then translates information of the detected ions into mass-to-charge ratios. Detection of the presence of a biomolecule or other substances will typically involve detection of signal intensity. This, in turn, can reflect the quantity and character of a biomolecule bound to the probe.

In another embodiment, the mass profile of a sample may be generated using a liquid chromatography (LC) based assay in which the biomolecules of a given sample are bound by biochemical or affinity interactions to an adsorbent located in a vessel made of glass, steel, or synthetic material known to those skilled in the art as a chromatography column. The biomolecules are eluted from the biologically active surface by washing the vessel with appropriate solutions known to those skilled in the art. Such solutions include but are not limited to, buffers, e.g. Tris (hydroxymethyl) aminomethane hydrochloride (TRIS-HCl), buffers containing salt, e.g. sodium chloride (NaCl), or organic solvents, e.g. acetonitrile. Biomolecule mass profiles are generated by application of the eluting biomolecules of the sample by direct connection via an electrospray device to a mass spectrometer (LC/ESI-MS).

Conditions that promote binding of biomolecules to an adsorbent are known to those skilled in the art (reference) and ordinarily include parameters such as pH, the concentration of salt, organic solvent, or other competitors for binding of the biomolecule to the adsorbent. Within the scope of the invention, incubation temperatures are of at least 0 to 100°C, preferably of at least 4 to 60°C, and most preferably of at least 15 to 30°C. Varying additional parameters, such as incubation time, the concentration of detergent, e.g. 3-[(3-Cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPS), or reducing agents, e.g. dithiothreitol (DTT), are also known to those skilled in the art. Various degrees of binding can be accomplished by combining the above stated conditions as needed, and will be readily apparent to those skilled in the art.

f) Methods for detecting biomolecules within a sample

In yet another aspect, the invention relates to methods for detecting differentially present biomolecules in a test sample and/or biological sample. Within the context of the invention, any suitable method can be used to detect one or more of the biomolecules described herein. For example, gas phase ion spectrometry can be used. This technique includes, e.g., laser desorption/ionization mass spectrometry. Preferably, the test and/or biological sample is prepared prior to gas phase ion spectrometry, e.g., ion fractionation, two dimensional gel chromatography, high performance liquid chromatography, etc. to assist detection of said biomolecules. Detection of said biomolecules can also be achieved using methods other than gas phase ion spectrometry. For example, immunoassays can be used to detect the biomolecules within a sample.

In one embodiment, the test and/or biological sample is prepared prior to contacting a biologically active surface and is in aqueous form. Examples said samples include, but are not limited to, blood, blood serum, plasma, nipple aspirate, urine, semen, seminal fluid, seminal plasma, prostatic fluid, feces, saliva, sweat, ascites, cerebrospinal fluid, milk, lymph, or tissue extract samples. Furthermore, solid test and/or biological samples, such as excreta or biopsy samples can be solubilized in or admixed with an eluent using methods known to those skilled in the art such that said samples may be

easily applied to a biologically active surface. Test and/or biological samples in the aqueous form can be further prepared using specific solutions for denaturation (pre-treatment) like sodium dodecyl sulfate, mercaptoethanol, urea, etc. For example, a test and/or biological sample of the invention can be denatured prior to contacting a biologically active surface comprising of quaternary ammonium groups by diluting said sample 1:5 with a buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT and 2% ampholine.

The sample is contacted with a biologically active surface using any techniques including bathing, soaking, dipping, spraying, washing over, or pipetting, etc. Generally, a volume of sample containing from a few atomoles to 100 picomoles of a biomolecule in about 1 to 500 µl is sufficient for detecting binding of the biomolecule to the adsorbent.

The pH value of the solvent in which the sample contacts the biologically active surface is a function of the specific sample and the selected biologically active surface. Typically, a sample is contacted with a biologically active surface under pH values between 0 and 14, preferably between about 4 and 10, more preferably between 4.5 and 9.0, and most preferably, at pH 8.5. The pH value depends on the type of adsorbent present on a biologically active surface and can be adjusted accordingly.

The sample can contact the adsorbent present on a biologically active for a period of time sufficient to allow the marker to bind to the adsorbent. Typically, the sample and the biologically active surface are contacted for a period of between about 1 second and about 12 hours, preferably, between about 30 seconds and about 3 hours, and most preferably for 120 minutes.

The temperature at which the sample contacts the biologically active surface (incubation temperature) is a function of the specific sample and the selected biologically active surface. Typically, the washing solution can be at a temperature of between 0 and 100°C, preferably between 4 and 37°C, and most preferably between 20 and 24°C.

For example, a biologically active surface comprising of quaternary ammonium groups (anion exchange surface) will bind the biomolecules described herein when the pH value is between 6.5 and 9.0. Optimal binding of the biomolecules of the present invention occurs at a pH of 8.5. Furthermore, a sample is contacted with said biologically active surface for 120 min. at a temperature of 20 – 24 °C.

Following contacting a sample or sample solution with a biological surface, it is preferred to remove any unbound biomolecules so that only the bound biomolecules remain on the biologically active surface. Washing unbound biomolecules are removed by methods known to those skilled in the art such as bathing, soaking, dipping, rinsing, spraying, or washing the biologically active surface with an

diner as a washing solution. A microfluidics process is preferably used when a washing solution such as an eluent is introduced to small spots of adsorbents on the biologically active surface. Typically, the washing solution can be at a temperature of between 0 and 100°C, preferably between 4 and 37°C, and more preferably between 20 and 37°C.

Washing solution or eluents used to wash the unbound biomolecules from a biologically active surface include, but are not limited to, organic solutions, aqueous solutions such as buffers wherein a buffer may contain detergents, salts, or chelating agents in concentrations as those known to those skilled in the art.

Aqueous solutions are preferred for washing biologically active surfaces. Exemplary aqueous solutions include, but are limited to, HEPES buffer, Tris buffer, phosphate buffered saline (PBS), and modifications thereof. The selection of a particular washing solution or eluent is dependent on other experimental conditions (e.g. types of adsorbents used or biomolecules of the interest), and can be determined by those of skill in the art. For example, if a biologically active surface comprising a quaternary ammonium group as adsorbent (anion exchange surface) is used, then an aqueous solution, such as a Tris buffer, may be preferred. In another example, if a biologically active surface comprising a carboxylate group as adsorbent (cation exchange surface) is used, then an aqueous solution, such as an acetate buffer, may be preferred.

Optionally, an energy absorbing molecule (EAM), e.g. in solution, can be applied to biomolecules or other substances bound on the biologically active surface by spraying, pipetting or dipping. Applying an EAM can be done after unbound substances are washed off of the biologically active surface. Exemplary energy absorbing molecules include, but are not limited to, cinnamic acid derivatives, sinapinic acid and dihydroxybenzoic acid.

Once the biologically active surface is free of any unbound biomolecules, adsorbent bound biomolecules are desorbed into gas phase for spectrometry. The quality and characteristics of a biomolecule can be determined using desorption, ionization and biomolecules can be analyzed using a gas phase ion spectrometer such as mass spectrometer, ion mobility spectrometer, or multiple ion mobility spectrometers. Other gas phase ion spectrometers known to those skilled in the art are also included.

In one embodiment, mass spectrometry can be used to detect biomolecules of a given sample present on a biologically active surface. Such methods include, but are not limited to, matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF), surface-enhanced laser desorption ionization/time-of-flight (SELDI-TOF), liquid chromatography coupled with MS, MS/MS, or

ESI-MS. Typically, biomolecules are analyzed by introducing a biologically active surface containing said biomolecules, ionizing said biomolecules to generate ions that are collected and analyzed.

In a preferred embodiment, the biomolecules present in a sample are detected using gas phase ion spectrometry, and more preferably, using mass spectrometry. In one embodiment, matrix-assisted laser desorption/ionization ("MALDI") mass spectrometry can be used. In MALDI, the sample is typically quasi-purified to obtain a fraction that essentially consists of a marker using separation methods such as two-dimensional gel electrophoresis or high performance liquid chromatography (HPLC).

In another embodiment, surface-enhanced laser desorption/ionization mass spectrometry ("SELDI") can be used. SELDI uses a substrate comprising adsorbents to capture biomolecules, which can then be directly detected and ionized from the substrate surface during mass spectrometry. Since the substrate surface in SELDI captures biomolecules, a sample need not be quasi-purified as in MALDI. However, depending on the complexity of a sample and the type of adsorbents used, it may be desirable to prepare a sample to reduce its complexity prior to SELDI analysis.

For example, biomolecules bound to a biologically active surface can be introduced into an inlet system of the mass spectrometer. The biomolecules are then ionized by an ionization source such as a laser, fast atom bombardment, or plasma. The generated ions are then collected by an ion optic assembly, and then a mass analyzer disperses the passing ions. The ions exiting the mass analyzer are detected by a detector and translated into mass-to-charge ratios. Detection of the presence of a biomolecule typically involves detection of its specific signal intensity, and reflects the quantity and character of said biomolecule.

In a preferred embodiment, a laser desorption time-of-flight mass spectrometer is used with the probe of the present invention. In laser desorption mass spectrometry, biomolecules bound to a biologically active surface are introduced into an inlet system. Biomolecules are desorbed and ionized into the gas phase by a laser. The ions generated are then collected by an ion optic assembly. These ions are accelerated through a short high voltage field and let drift into a high vacuum chamber of a time-of-flight mass analyzer. At the far end of the high vacuum chamber, the accelerated ions strike a sensitive detector surface at a different time. Since the time-of-flight is a function of the mass of the ions, the elapsed time between ionization and impact can be used to identify the presence or absence of molecules of a specific mass.

The detection of biomolecules described herein can be enhanced using certain selectivity conditions (i. e. type of adsorbents used or washing solutions). In a preferred embodiment, the same or substantially the same selectivity conditions that were used to discover the biomolecules can be used

in the methods for detecting a biomolecule in a sample.

Combinations of the laser desorption time-of-flight mass spectrometer with other components described herein, in the assembly of mass spectrometer that employs various means of desorption, acceleration, detection, measurement of time, etc., are known to those skilled in the art.

Data generated by desorption and detection of markers can be analyzed with the use of a programmable digital computer. The computer program generally contains a readable medium that stores codes. Certain codes can be devoted to memory that include the location of each feature on a biologically active surface, the identity of the adsorbent at that feature and the elution conditions used to wash the adsorbent. Using this information, the program can then identify the set of features on the biologically active surface defining certain selectivity characteristics (e. g. types of adsorbent and eluents used). The computer also contains codes that receive as data (input) on the strength of the signal at various molecular masses received from a particular addressable location on the biologically active surface. This data can indicate the number of biomolecules detected, as well as the strength of the signal and the determined molecular mass for each biomolecule detected.

Data analysis can include the steps of determining signal strength (e. g., height of peaks) of a biomolecule detected and removing "outliers" (data deviating from a predetermined statistical distribution). For example, the observed peaks can be normalized, a process whereby the height of each peak relative to some reference is calculated. For example, a reference can be background noise generated by instrument and chemicals (e. g., energy absorbing molecule), which is set as zero in the scale. Then the signal strength detected for each biomolecule can be displayed in the form of relative intensities in the scale desired (e. g., 100). Alternatively, a standard may be admitted with the sample so that a peak from the standard can be used as a reference to calculate relative intensities of the signals observed for each biomolecule or other biomolecules detected.

The computer can transform the resulting data into various formats for displaying. In one format, referred to as "spectrum view", a standard spectral view can be displayed, wherein the view depicts the quantity of a biomolecule reaching the detector at each particular molecular mass. In another format, referred to as "scatter plot" only the peak height and mass information are retained from the spectrum view, yielding a cleaner image and enabling biomolecules with nearly identical molecular mass to be more visible.

Using any of the above display formats, it can be readily determined from the signal display whether a biomolecule having a particular molecular mass is detected from a sample. Preferred biomolecules of the invention are biomolecules with an apparent molecular mass of about 2020 Da ± 10 Da, 2049 Da ± 10 Da, 2270 Da ± 11 Da, 2508 Da ± 13 Da, 2732 Da ± 14 Da, 3026 Da ± 15 Da, 3227 Da ± 17 Da, 3326 Da ± 17 Da, 3456 Da ± 17 Da, 3946 Da ± 20 Da, 4103 Da ± 21 Da, 4242 Da ± 21 Da, 4295 Da ± 21 Da, 4359 Da ± 22 Da, 4476 Da ± 22 Da, 4546 Da ± 23 Da, 4607 Da ± 23 Da, 4719 Da ± 24 Da, 4830 Da ± 24 Da, 4865 Da ± 24 Da, 4963 Da ± 25 Da, 5112 Da ± 26 Da, 5226 Da ± 26 Da, 5493 Da ± 27 Da, 5648 Da ± 28 Da, 5772 Da ± 29 Da, 5854 Da ± 29 Da, 6446 Da ± 32 Da, 6644 Da ± 33 Da, 6852 Da ± 34 Da, 6897 Da ± 34 Da, 6999 Da ± 35 Da, 7575 Da ± 38 Da, 7657 Da ± 38 Da, 8076 Da ± 40 Da, 8215 Da ± 41 Da, 8474 Da ± 42 Da, 8574 Da ± 43 Da, 8702 Da ± 44 Da, 8780 Da ± 44 Da, 8922 Da ± 45 Da, 9078 Da ± 45 Da, 9143 Da ± 46 Da, 9201 Da ± 46 Da, 9359 Da ± 47 Da, 9425 Da ± 47 Da, 9581 Da ± 48 Da, 9641 Da ± 48 Da, 9718 Da ± 49 Da, 9930 Da ± 50 Da, 10215 Da ± 51 Da, 10369 Da ± 52 Da, 10440 Da ± 52 Da, 10594 Da ± 53 Da, 11216 Da ± 56 Da, 11464 Da ± 57 Da, 11547 Da ± 58 Da, 11693 Da ± 58 Da, 11905 Da ± 60 Da, 12470 Da ± 62 Da, 12619 Da ± 63 Da, 12828 Da ± 64 Da, 13290 Da ± 66 Da, 13632 Da ± 68 Da, 13784 Da ± 69 Da, 13983 Da ± 70 Da, 14798 Da ± 74 Da, 15005 Da ± 75 Da, 15140 Da ± 76 Da, 15350 Da ± 77 Da, 15879 Da ± 79 Da, 15957 Da ± 80 Da, 16104 Da ± 81 Da, 16164 Da ± 81 Da, 16953 Da ± 85 Da, 17263 Da ± 86 Da, 17397 Da ± 87 Da, 17617 Da ± 88 Da, 17766 Da ± 89 Da, 17890 Da ± 89 Da, 18115 Da ± 91 Da, 18390 Da ± 92 Da, 22338 Da ± 112 Da, 22466 Da ± 112 Da, 22676 Da ± 113 Da, 22951 Da ± 115 Da, 24079 Da ± 120 Da, 28055 Da ± 140 Da, or 28259 Da ± 141 Da. Moreover, from the strength of signal, the amount of a biomolecule bound on the biologically active surface can be determined.

g) Identification of proteins

In case the biomolecules of the invention are proteins, the present invention comprises a method for the identification of these proteins, especially by obtaining their amino acid sequence. This method comprises the purification of said proteins from the complex biological sample (blood, blood serum, plasma, nipple aspirate, urine, semen, seminal fluid, seminal plasma, prostatic fluid, tears, saliva, sweat, ascites, cerebrospinal fluid, milk, lymph, or tissue extract samples) by fractionating said sample using techniques known by the one of ordinary skill in the art, most preferably protein chromatography (FPLC, HPLC).

The biomolecules of the invention include those proteins with a molecular mass selected from 2020 Da ± 10 Da, 2049 Da ± 10 Da, 2270 Da ± 11 Da, 2508 Da ± 13 Da, 2732 Da ± 14 Da, 3026 Da ± 15 Da, 3227 Da ± 17 Da, 3326 Da ± 17 Da, 3456 Da ± 17 Da, 3946 Da ± 20 Da, 4103 Da ± 21 Da, 4242 Da ± 21 Da, 4295 Da ± 21 Da, 4359 Da ± 22 Da, 4476 Da ± 22 Da, 4546 Da ± 23 Da, 4607 Da ± 23 Da, 4719 Da ± 24 Da, 4830 Da ± 24 Da, 4865 Da ± 24 Da, 4963 Da ± 25 Da, 5112 Da ± 26 Da, 5226 Da ± 26 Da, 5493 Da ± 27 Da, 5648 Da ± 28 Da, 5772 Da ± 29 Da, 5854 Da ± 29 Da, 6446 Da ± 32 Da, 6644 Da ± 33 Da, 6852 Da ± 34 Da, 6897 Da ± 34 Da, 6999 Da ± 35 Da, 7575 Da ± 38 Da, 7657 Da ± 38 Da, 8076 Da ± 40 Da, 8215 Da ± 41 Da, 8474 Da ± 42 Da, 8574 Da ± 43 Da, 8702 Da ± 44 Da, 8780 Da ± 44 Da, 8922 Da ± 45 Da, 9078 Da ± 45 Da, 9143 Da ± 46 Da, 9201 Da ± 46 Da, 9359

Da ± 47 Da, 9425 Da ± 47 Da, 9581 Da ± 48 Da, 9641 Da ± 48 Da, 9718 Da ± 49 Da, 9930 Da ± 50 Da, 10215 Da ± 51 Da, 10369 Da ± 52 Da, 10440 Da ± 52 Da, 10594 Da ± 53 Da, 11216 Da ± 56 Da, 11464 Da ± 57 Da, 11547 Da ± 58 Da, 11693 Da ± 58 Da, 11905 Da ± 60 Da, 12470 Da ± 62 Da, 12619 Da ± 63 Da, 12828 Da ± 64 Da, 13290 Da ± 66 Da, 13632 Da ± 68 Da, 13784 Da ± 69 Da, 13983 Da ± 70 Da, 14798 Da ± 74 Da, 15005 Da ± 75 Da, 15140 Da ± 76 Da, 15350 Da ± 77 Da, 15879 Da ± 79 Da, 15957 Da ± 80 Da, 16104 Da ± 81 Da, 16164 Da ± 81 Da, 16953 Da ± 85 Da, 17263 Da ± 86 Da, 17397 Da ± 87 Da, 17617 Da ± 88 Da, 17766 Da ± 89 Da, 17890 Da ± 89 Da, 18115 Da ± 91 Da, 18390 Da ± 92 Da, 22338 Da ± 112 Da, 22466 Da ± 112 Da, 22676 Da ± 113 Da, 22951 Da ± 115 Da, 24079 Da ± 120 Da, 28055 Da ± 140 Da, and 28259 Da ± 141 Da.

Furthermore, the method comprises the analysis of the fractions for the presence and purity of said proteins by the method which was used to identify them as differentially expressed biomolecules, for example two-dimensional gel electrophoresis or SELDI mass spectrometry, but most preferably MALDI mass spectrometry. The method also comprises an analysis of the purified proteins aiming towards the revealing of their amino acid sequence. This analysis may be performed using techniques in mass spectrometry known to those skilled in the art.

In one embodiment, this analysis may be performed using peptide mass fingerprinting, revealing information about the specific peptide mass profile after proteolytic digestion of the investigated protein.

In another embodiment, this analysis may be preferably performed using post-source-decay (PSD), or MS/MS, but most preferably MS/MS, revealing mass information about all possible fragments of the investigated protein or proteolytic peptides thereof leading to the amino acid sequence of the investigated protein or proteolytic peptide thereof.

The information revealed by the above-mentioned techniques can be used to feed world-wide-web search engines, such as MS FIT (Protein Prospector, http://prospector.ucsf.edu) for information obtained from peptide mass fingerprinting, or MS Tag (Protein Prospector, http://prospector.ucsf.edu) for information obtained from PSD, or mascot (www.matrixscience.com) for information obtained from MS/MS and peptide mass fingerprinting, for the alignment of the obtained results with data available in public protein sequence databases, such as SwissProt (http://www.expasy.org/sprot), NCBI (http://www.ncbi.nlm.nih.gov/BLAST/), EMBL (http://www.embl-heidelberg.de:8000/srs5/) which leads to a confident information about the identity of said protein.

This information may comprise, if available, the complete amino acid sequence, the calculated molecular mass, the structure, the enzymatic activity, the physiological function, and gene expression of the investigated proteins.

b) Kits

In yet another aspect, the invention provides kits using the methods of the invention as described in the section Diagnostics for the differential diagnosis of colorectal cancer or a non-malignant disease of the large intestine, wherein the kits are used to detect the biomolecules of the present invention.

The methods used to detect the biomolecules of the invention can also be used to determine whether a subject is at risk of developing colorectal cancer or a non-malignant disease of the large intestine, or has developed a colorectal cancer or a non-malignant disease of the large intestine. Such methods may also be employed in the form of a diagnostic kit comprising an antibody specific to a biomolecule of the invention or a biologically active surface described herein, which may be conveniently used, for example, in clinical settings to diagnose patients exhibiting symptoms or a family history of a non-steroid dependent cancer. Such diagnostic kits also include solutions and materials necessary for the detection of a biomolecule of the invention, and instructions to use the kit based on the above-mentioned methods.

The biomolecules of the invention include those proteins with a molecular mass selected from 2020 Da ± 10 Da, 2049 Da ± 10 Da, 2270 Da ± 11 Da, 2508 Da ± 13 Da, 2732 Da ± 14 Da, 3026 Da ± 15 Da, 3227 Da ± 17 Da, 3326 Da ± 17 Da, 3456 Da ± 17 Da, 3946 Da ± 20 Da, 4103 Da ± 21 Da, 4242 Da ± 21 Da, 4295 Da ± 21 Da, 4359 Da ± 22 Da, 4476 Da ± 22 Da, 4546 Da ± 23 Da, 4607 Da ± 23 Da, 4719 Da ± 24 Da, 4830 Da ± 24 Da, 4865 Da ± 24 Da, 4963 Da ± 25 Da, 5112 Da ± 26 Da, 5226 Da ± 26 Da, 5493 Da ± 27 Da, 5648 Da ± 28 Da, 5772 Da ± 29 Da, 5854 Da ± 29 Da, 6446 Da ± 32 Da, 6644 Da ± 33 Da, 6857 Da ± 34 Da, 6897 Da ± 34 Da, 6999 Da ± 35 Da, 7575 Da ± 38 Da, 7657 Da ± 38 Da, 8076 Da ± 40 Da, 8215 Da ± 41 Da, 8474 Da ± 42 Da, 8574 Da ± 43 Da, 8702 Da ± 44 Da, 8780 Da ± 44 Da, 8922 Da ± 45 Da, 9078 Da ± 45 Da, 9143 Da ± 46 Da, 9201 Da ± 46 Da, 9359 Da ± 47 Da, 9425 Da ± 47 Da, 9581 Da ± 48 Da, 9641 Da ± 48 Da, 9718 Da ± 49 Da, 9930 Da ± 50 Da, 10215 Da ± 51 Da, 10369 Da ± 52 Da, 10440 Da ± 52 Da, 10594 Da ± 53 Da, 11216 Da ± 56 Da, 11464 Da ± 57 Da, 11547 Da ± 58 Da, 11685 Da ± 58 Da, 11985 Da ± 60 Da, 12470 Da ± 62 Da, 12619 Da ± 63 Da, 12828 Da ± 64 Da, 13290 Da ± 66 Da, 13632 Da ± 68 Da, 13784 Da ± 69 Da, 13983 Da ± 70 Da, 14798 Da ± 74 Da, 15005 Da ± 75 Da, 15140 Da ± 76 Da, 15350 Da ± 77 Da, 15879 Da ± 79 Da, 15957 Da ± 80 Da, 16104 Da ± 81 Da, 16164 Da ± 81 Da, 16953 Da ± 85 Da, 17263 Da ± 86 Da, 17397 Da ± 87 Da, 17617 Da ± 88 Da, 17766 Da ± 89 Da, 17890 Da ± 89 Da, 18115 Da ± 91 Da, 18390 Da ± 92 Da, 22338 Da ± 112 Da, 22466 Da ± 112 Da, 22676 Da ± 113 Da, 22951 Da ± 115 Da, 24079 Da ± 120 Da, 28055 Da ± 140 Da, or 28259 Da ± 141 Da.

For example, the kits can be used to detect one or more of differentially present biomolecules as described above in a test sample of subject. The kits of the invention have many applications. For example, the kits can be used to differentiate if a subject is healthy, having a precancerous lesion of the large intestine, a colorectal cancer, a retarded colorectal cancer or a non-malignant disease of the large intestine. Thus aiding the diagnosis of colorectal cancer or a non-malignant disease of the large intestine. In another example, the kits can be used to identify compounds that modulate expression of said biomolecules.

In one embodiment, a kit comprises an adsorbent on a biologically active surface, wherein the adsorbent is suitable for binding one or more biomolecules of the invention, a denaturation solution for the pre-treatment of a sample, a binding solution, a washing solution or instructions for making a denaturation solution, binding solution, or washing solution, wherein the combination allows for the detection of a biomolecule using gas phase ion spectrometry. Such kits can be prepared from the materials described in other previously detailed sections (e. g., denaturation buffer, binding buffer, adsorbents, washing solutions, etc.).

In some embodiments, the kit may comprise a first substrate comprising an adsorbent thereon (e.g., a particle functionalized with an adsorbent) and a second substrate onto which the first substrate can be positioned in first a probe, which is interrogable by laser desorption spectrometer. In other embodiments, the kit may comprise a single substrate, which is in the form of a removably insertable probe with adsorbent on the substrate.

In another embodiment, a kit comprises a binding molecule that specifically binds to a biomolecule related to the invention, a detection reagent, appropriate sample(s) and instructions on how to use the kit. Such kits can be prepared from the materials described above, and other materials known to those skilled in the art. A binding molecule used within such a kit may include, but is not limited to, proteins, peptides, nucleotides, nucleic acids, hormones, amino acids, sugars, fatty acids, steroids, polysaccharides, carbohydrates, lipids, or a combination thereof (e.g., glycoproteins, ribonucleoproteins, lipoproteins), compounds or synthetic molecules. Preferably, a binding molecule specifically binds an antibody.

In other embodiments, the kit may optionally further comprise a standard or control information so that the test sample can be compared with the control information standard to determine if the test amount of a marker detected in a sample is a diagnostic amount consistent with a diagnosis of colorectal cancer.

The present invention also relates to use 2020 Da ± 10 Da, 2049 Da ± 10 Da, 2270 Da ± 11 Da, 2508 Da ± 13 Da, 2732 Da ± 14 Da, 3026 Da ± 15 Da, 3227 Da ± 17 Da, 3326 Da ± 17 Da, 3456 Da ± 17 Da, 3946 Da ± 20 Da, 4103 Da ± 21 Da, 4242 Da ± 21 Da, 4295 Da ± 21 Da, 4359 Da ± 22 Da, 4476 Da ± 22 Da, 4546 Da ± 23 Da, 4607 Da ± 23 Da, 4719 Da ± 24 Da, 4830 Da ± 24 Da, 4865 Da ± 24 Da, 4963 Da ± 25 Da, 5112 Da ± 26 Da, 5226 Da ± 26 Da, 5493 Da ± 27 Da, 5648 Da ± 28 Da, 5772 Da ± 29 Da, 5854 Da ± 29 Da, 6446 Da ± 32 Da, 6644 Da ± 33 Da, 6852 Da ± 34 Da, 6897 Da ± 34 Da, 6999 Da ± 35 Da, 7375 Da ± 38 Da, 7657 Da ± 38 Da, 8076 Da ± 40 Da, 8215 Da ± 41 Da, 8474 Da ± 42 Da, 8574 Da ± 43 Da, 8702 Da ± 44 Da, 8780 Da ± 44 Da, 8922 Da ± 45 Da, 9078 Da ± 45 Da, 9143 Da ± 46 Da, 9281 Da ± 46 Da, 9359 Da ± 47 Da, 9425 Da ± 47 Da, 9581 Da ± 48 Da, 9641 Da ± 48 Da, 9718 Da ± 49 Da, 9930 Da ± 50 Da, 10215 Da ± 51 Da, 10369 Da ± 52 Da, 10440 Da ± 52 Da, 10594 Da ± 53 Da, 11216 Da ± 56 Da, 11464 Da ± 57 Da, 11547 Da ± 58 Da, 11693 Da ± 58 Da, 11905 Da ± 60 Da, 12470 Da ± 62 Da, 12619 Da ± 63 Da, 12828 Da ± 64 Da, 13290 Da ± 66 Da, 13632 Da ± 68 Da, 13784 Da ± 69 Da, 13983 Da ± 70 Da, 14798 Da ± 74 Da, 15005 Da ± 75 Da, 15140 Da ± 76 Da, 15350 Da ± 77 Da, 15879 Da ± 79 Da, 15997 Da ± 80 Da, 16104 Da ± 81 Da, 16164 Da ± 81 Da, 16953 Da ± 85 Da, 17263 Da ± 86 Da, 17397 Da ± 87 Da, 17617 Da ± 88 Da, 17766 Da ± 89 Da, 17890 Da ± 89 Da, 18115 Da ± 91 Da, 18390 Da ± 92 Da, 22338 Da ± 112 Da, 22468 Da ± 112 Da, 22676 Da ± 113 Da, 22951 Da ± 115 Da, 24079 Da ± 120 Da, 28055 Da ± 140 Da, or 28259 Da ± 141 Da for manufacture of an agent for diagnosis, prophylactic and/or therapeutic treatment of non-steroid dependent cancer, preferably colorectal cancer.

The invention also relates to a method for aiding non-steroid dependent cancer diagnosis especially colorectal cancer, the method comprising (a) detecting at least one protein marker in a sample, wherein the protein marker is selected from 2020 Da ± 10 Da, 2049 Da ± 10 Da, 2270 Da ± 11 Da, 2508 Da ± 13 Da, 2732 Da ± 14 Da, 3026 Da ± 15 Da, 3227 Da ± 17 Da, 3326 Da ± 17 Da, 3456 Da ± 17 Da, 3946 Da ± 20 Da, 4103 Da ± 21 Da, 4242 Da ± 21 Da, 4295 Da ± 21 Da, 4359 Da ± 22 Da, 4476 Da ± 22 Da, 4546 Da ± 23 Da, 4607 Da ± 23 Da, 4719 Da ± 24 Da, 4830 Da ± 24 Da, 4865 Da ± 24 Da, 4963 Da ± 25 Da, 5112 Da ± 26 Da, 5226 Da ± 26 Da, 5493 Da ± 27 Da, 5648 Da ± 28 Da, 5772 Da ± 29 Da, 5854 Da ± 29 Da, 6446 Da ± 32 Da, 6644 Da ± 33 Da, 6852 Da ± 34 Da, 6897 Da ± 34 Da, 6999 Da ± 35 Da, 7375 Da ± 38 Da, 7657 Da ± 38 Da, 8076 Da ± 40 Da, 8215 Da ± 41 Da, 8474 Da ± 42 Da, 8574 Da ± 43 Da, 8702 Da ± 44 Da, 8780 Da ± 44 Da, 8922 Da ± 45 Da, 9078 Da ± 45 Da, 9143 Da ± 46 Da, 9281 Da ± 46 Da, 9359 Da ± 47 Da, 9425 Da ± 47 Da, 9581 Da ± 48 Da, 9641 Da ± 48 Da, 9718 Da ± 49 Da, 9930 Da ± 50 Da, 10215 Da ± 51 Da, 10369 Da ± 52 Da, 10440 Da ± 52 Da, 10594 Da ± 53 Da, 11216 Da ± 56 Da, 11464 Da ± 57 Da, 11547 Da ± 58 Da, 11693 Da ± 58 Da, 11905 Da ± 60 Da, 12470 Da ± 62 Da, 12619 Da ± 63 Da, 12828 Da ± 64 Da, 13290 Da ± 66 Da, 13632 Da ± 68 Da, 13784 Da ± 69 Da, 13983 Da ± 70 Da, 14798 Da ± 74 Da, 15005 Da ± 75 Da, 15140 Da ± 76 Da, 15350 Da ± 77 Da, 15879 Da ± 79 Da, 15997 Da ± 80 Da, 16104 Da ± 81 Da, 16164 Da ± 81 Da, 16953 Da ± 85 Da, 17263 Da ± 86 Da, 17397 Da ± 87 Da, 17617 Da ± 88 Da,

17766 Da ± 89 Da, 17890 Da ± 89 Da, 18115 Da ± 91 Da, 18390 Da ± 92 Da, 22338 Da ± 112 Da, 22466 Da ± 112 Da, 22676 Da ± 113 Da, 22951 Da ± 115 Da, 24079 Da ± 120 Da, 28055 Da ± 140 Da, or 28259 Da ± 141 Da and (b) correlating the detection of the or protein marker with a probable diagnosis of non-steroid cancer especially colorectal cancer.

Each recorded measurement reading is accompanied by a margin of deviation. The latter statistical imprecision is well-known to those skilled in the art. In the scope of the present invention, the margin of deviation is exclusively device-specific. That means it is caused by the type of analytical device used which is preferably a mass spectrometer. The accuracy of the recorded measurement reading is specified by a fixed percentage. In the meaning of the present invention, each disclosed molecular mass represents the averaged value of that range which deviates from the averaged value about ± 0.5 %.

Furthermore, slight differences appear in the molecular mass value itself which concerns the same protein in parallel patent applications disclosing the matter of cancer biomarkers. There are three reasons to be considered. First, each molecular mass results from the analysis of samples belonging to another type of cancer. The origin of sample, the cellular status, the environmental conditions of the gathered tissue etc. exert an influence on the measurements. Secondly, the given molecular mass of the biomarkers represents the averaged value which is calculated from the data of numerous samples of each cancer species. Thirdly, measuring errors might be also imaginable, for example due to the sample preparation.

Above statements are further illustrated by examples which should not be construed as limiting with regard to the type of disease, the number of given molecular masses or in any other way. The following molecular masses of biomolecules are regarded as equivalent:

(i) 2020 ± 10 (epithelial cancer) and 2020 ± 10 (colorectal cancer)
(ii) 2050 ± 10 (epithelial cancer) and 2049 ± 10 (colorectal cancer)
(iii) 3946 ± 20 (epithelial cancer) and 3946 ± 20 (colorectal cancer)
(iv) 4104 ± 21 (epithelial cancer) and 4103 ± 21 (colorectal cancer)
(v) 4298 ± 21 (epithelial cancer) and 4295 ± 21 (colorectal cancer)
(vi) 4360 ± 22 (epithelial cancer) and 4359 ± 22 (colorectal cancer)
(vii) 4477 ± 22 (epithelial cancer) and 4476 ± 22 (colorectal cancer)
(viii) 4867 ± 24 (epithelial cancer) and 4865 ± 24 (colorectal cancer)
(ix) 4958 ± 25 (epithelial cancer) and 4963 ± 25 (colorectal cancer)

(x) 5491 ± 27 (epithelial cancer) and 5493 ± 27 (colorectal cancer)
(xi) 5650 ± 28 (epithelial cancer) and 5648 ± 28 (colorectal cancer)
(xii) 6449 ± 32 (epithelial cancer) and 6446 ± 32 (colorectal cancer)
(xiii) 6876 ± 34 (epithelial cancer) and 6852 ± 34 (colorectal cancer)
(xiv) 7001 ± 35 (epithelial cancer) and 6999 ± 35 (colorectal cancer)
(xv) 8232 ± 41 (epithelial cancer) and 8215 ± 41 (colorectal cancer)
(xvi) 8711 ± 44 (epithelial cancer) and 8702 ± 44 (colorectal cancer)
(xvii) 12471 ± 62 (epithelial cancer) and 12470 ± 62 (colorectal cancer)
(xviii) 12669 ± 63 (epithelial cancer) and 12619 ± 63 (colorectal cancer)
(xix) 13989 ± 70 (epithelial cancer) and 13983 ± 70 (colorectal cancer)
(xx) 15959 ± 80 (epithelial cancer) and 15957 ± 80 (colorectal cancer)
(xxi) 16164 ± 81 (epithelial cancer) and 16164 ± 81 (colorectal cancer)
(xxii) 17279 ± 86 (epithelial cancer) and 17263 ± 86 (colorectal cancer)
(xxiii) 17406 ± 87 (epithelial cancer) and 17397 ± 87 (colorectal cancer)
(xxiv) 17630 ± 88 (epithelial cancer) and 17617 ± 88 (colorectal cancer)
(xxv) 18133 ± 91 (epithelial cancer) and 18115 ± 91 (colorectal cancer)

In all examples, each recorded measurement reading is overlapping with any others within its margin of deviation.

A further calculation of averaged values which incorporates the matching molecular masses of each type of cancer is known to those skilled in the art. By applying formulas which the method of error calculation by means of weights (weighted average) is based upon, the following generalized results are obtained for the aforementioned examples:

(i) 2020 ± 10
(ii) 2050 ± 10
(iii) 3946 ± 20
(iv) 4104 ± 21
(v) 4297 ± 21
(vi) 4360 ± 22
(vii) 4477 ± 22
(viii) 4866 ± 24

(ix) 4961 ± 25
(x) 5497 ± 27
(xi) 5870 ± 28
(xii) 6448 ± 33
(xiii) 6864 ± 34
(xiv) 7000 ± 35
(xv) 8224 ± 41
(xvi) 9707 ± 44
(xvii) 12471 ± 62
(xviii) 12644 ± 63
(xix) 13986 ± 70
(xx) 15958 ± 80
(xxi) 16164 ± 81
(xxii) 17271 ± 86
(xxiii) 17403 ± 87
(xxiv) 17624 ± 88
(xxv) 18124 ± 91

The present invention is further illustrated by the following examples, which should not be construed as limiting in any way. The contents of all cited references (including literature references, issued patents, published patent applications), as cited throughout this application, are hereby expressly incorporated by reference. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are known to those skilled in the art. Such techniques are explained fully in the literature.

Example 1: Sample collection for colon cancer evaluation.

Serum samples were obtained from a total of 151 individuals, which included two different groups of subjects. In the first group (group I), sera were drawn from 77 colon cancer patients undergoing diagnosis and treatment of colon cancer at the Departments of Gastroenterology and Surgery of the Universities of Magdeburg, Erlangen and Cottbus (all Germany). Serum samples were collected from the patients directly before surgery. At this time, a primary diagnosis was made based on endoscopy, ultrasound testing, and/or other means for the detection of colorectal cancer. In all cases the diagnosis was confirmed by histological evaluation after surgery. Follow-up data for all colon cancer patients are currently collected and will be available for later studies.

The non-cancer control group (group II) consisted of 94 subjects with non-malignant disease symptoms of the large intestine (adenoma, inflammation, diverticulosis), which were recruited from the University Hospitals in Magdeburg, Cottbus, and Erlangen. Serum from each subject was taken following colorectal endoscopy, wherein the absence of colorectal cancer was confirmed. Furthermore, all subjects denied a personal history of cancer and were otherwise healthy. Follow-up data for all non-cancer controls are currently collected and will be available for later studies. In addition, 77 serum samples from healthy blood donors was also collected for test-set analysis. Blood donors are considered to be healthy individuals not suffering from severe diseases.

Example 2: ProteinChip Array analysis.

ProteinChip Arrays of the SAX2-type (strong anion exchanger) were arranged into a bioprocessor (Ciphergen Biosystems, Inc.), a device that contains up to 12 ProteinChips and facilitates processing of the ProteinChips. The ProteinChips were pre-incubated in the bioprocessor with 200 µl binding buffer (0.1 M Tris-HCl, 0.02% Triton X-100, pH 8.5). 10 µl of serum sample was diluted 1:5 in a buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% ampholine) and again diluted 1:10 in the binding buffer. Then, 200 µl of this mixture (equivalent to 6 µl original serum sample) were directly applied onto the spots of the SAX2 ProteinChips. In between dilution steps and prior to the application to the spots, the sample was kept on ice (at 0°C). After incubation for 120 minutes at 20 to 24 °C the chips were incubated with 200 µl binding buffer, before 2 x 0.5 µl EAM solution (20 mg/ml sinapinic acid in 50% acetonitrile and 0.5% trifluoroacetic acid) was applied to the spots. After air-drying for 10 min, the ProteinChips were placed in the ProteinChip Reader (ProteinChip Biology System II, Ciphergen Biosystems, Inc.) and time-of-flight spectra were generated by laser shots collected in the positive mode at laser intensity 215, with the detector sensitivity of 8. Sixty laser shots per average spectra were performed.

Calibration of mass accuracy was performed by using the following mixture of mass standard calibrant proteins: Dynorphin A (porcine, 209 – 225, 2147.50 Da), Beta-endorphin (human, 61 – 91, 3465.00 Da), Insulin (bovine, 5733.51 Da), and Cytochrome c (bovine, 12230.30 Da) at a concentration of 1.21 pmol/µl, and Myoglobin (equine cardiac, 16951.50 Da) at a concentration of 5.16 pmol/µl. 0.5µl of this mixture was applied to a single spot of a H4 ProteinChip array. After air-drying of the drop, 2 x 1 µl matrix solution (a saturated solution of sinapinic acid in 50% acetonitrile 0.5% trifluoroacetic acid) was applied to the spot. The drop was allowed to air-dry for 10 min after each application of matrix solution.

The ProteinChip was placed in the ProteinChip Reader (Biology System II, Ciphergen Biosystems, Inc.) and time-of-flight spectra were generated by laser shots collected in the positive mode at laser

intensity 210, with the detector sensitivity at 8. Sixty laser shots per average spectra were performed. Subsequently, Time-Of-Flight values were correlated to the molecular masses of the standard proteins, and calibration was performed according to the instrument manual.

Example 3, Peak detection and data analysis:

The analysis of the data was performed by automatic peak detection and alignment using the operating software of the ProteinChip Biology System II, the ProteinChip Software Version 3.1 (Ciphergen Biosystems, Inc.). Figure 1 shows a comparison of protein mass spectra detected using the above mentioned SELDI ProteinChip array for samples isolated from patients suffering from non-malignant diseases of the colon mucosa (e.g., acute or chronic inflammation, adenoma) (G1 and G2) and of patients with colon cancer (T1 and T2).

The complete set of patients was randomly divided into a training set and a test set. The train set comprised of 54 randomly selected patients with colon cancer and 73 randomly selected patients without colon cancer. The test set comprised of 14 randomly selected patients with colon cancer and 19 randomly selected patients without colon cancer. Additionally, a test set comprising of 77 samples obtained from healthy blood donors was compiled. This was done in order to test the classification algorithm generated on the basis of the spectra of the subgroup of healthy individuals (see below).

The m/z values of all mass spectra selected for the analysis ranged between 2000 Da and 20000 Da, wherein smaller masses were not used since artifacts with the "Energy Absorbing Molecule, HAM" ("Matrix") could not be excluded, and higher masses were not detected under the chosen experimental conditions. The spectra within the train set were normalized according to the intensity of the total ion current, followed by baseline subtraction, and automatic peak detection as previously described by Adam, B. et al. (2002) Cancer Research 62: 3609-3614, using the "Biomarker Wizard" tool of the ProteinChip Software Version 3.1 (Ciphergen Biosystems, Inc.). The following settings were chosen for peak detection by "Biomarker Wizard": a) auto-detect peaks in cluster, b) first pass: 5 spots/cluster, c) minimum peak threshold: 5% of all spectra, d) deletion of auto-detected peaks below threshold, e) cluster mass window: 400.3% of mass. Using these settings, 95 signal clusters were identified.

The normalization coefficient generated by normalizing the spectra of the train sets and the cluster information of the train sets generated by the "Biomarker Wizard" tool of the software were saved and used to externally normalize the spectra of the corresponding test sets and to cluster the signals of the corresponding test sets according to the identification and peak identification of the train sets.

The cluster information for each train and test set (containing sample ID and sample group, cluster mass values and cluster signal intensities for each spectrum within the set) was transformed into an

interchangeable data format (a .csv table) using the "Sample group statistics" function of the "Biomarker Wizard" tool of the ProteinChip Software Version 3.1. In this format, the data can be analyzed by a specific software for the generation of regression and classification trees (see examples 5 to 7).

Example 4, Construction of classifiers.

Four classifiers with binary target variable (cancer versus non-cancer) were constructed: First, as a proof of principle, a classifier was constructed only on the basis of the training set described above. Second, a final classifier was constructed on the basis of all available mass peaks and all colon cancer samples, fusing the corresponding training and test data sets. Third, a 2nd final colon classifier was constructed analogously to the first final colon cancer classifier but excluding the most informative and dominating mass of the first final colon classifier. Fourth, a 3rd final colon classifier was constructed analogously to the first final colon cancer classifier but excluding the most informative and dominating masses of the first and 2nd final colon classifier.

Forward variable selection was applied in order to determine highly informative sets of variables ("peaks") for classification. The results of the present invention were generated using the "CART" decision tree approach (classification and regression trees; Breiman et al., 1984). Moreover, bagging of classifiers was applied to overcome typical instabilities of forward variable selection procedures, thereby increasing overall classifier performance (Breiman, 1996).

More precisely, for the training set 50 bootstrap samples were generated (sampling with replacement, minimal 5 sample recovery). For each bootstrap sample an exploratory decision tree was generated. Nodes were split using the Gini rule until all final nodes were either pure, i.e., contained only samples of one class, or until one of the following stopping rules was met: no nodes comprising less than 4 cases were split and no splits were considered resulting in a node comprising only one sample. The each obtained 50 single classifiers, one for each bootstrap sample, were combined to constitute an ensemble of classifiers predicting class membership by plurality vote.

The procedure of classifier construction was conducted four times to obtain one proof-of-principle classifier and three final classifiers for colon cancer detection.

Example 5, Classifier structure.

The proof-of-principle classifier employed 71 masses (variables) out of 90 determined signal clusters. Single decision trees consisted of 4 to 9 variables (5 to 10 end nodes), 6 variables being typical, see histogram of Figure 4. Variable importance was roughly deduced by overall improvement, i.e., for each mass we summed the improvement values achieved in the generation of all 50 decision trees of

the decision tree ensemble. The masses used by the proof-of-principle classifier are listed in Table 1 (starting with most important masses having high improvement). An overview of the distribution of masses is given in Figure 5.

The 1st final classifier for colon cancer employed 75 masses out of 90 determined signal clusters. Single decision trees consisted of more variables than in the proof-of-principle classifier 9 variables were typical, see histogram of Figure 6. Variable importance was roughly deduced by overall improvement. The masses used by the 1st final classifier are listed in Table 2 (starting with most important masses, i.e. masses with highest improvement values). An overview of the distribution of masses of the 1st final classifier is given in Figure 7.

The 2nd final classifier for colon cancer employed 77 masses out of 90 determined signal clusters. Single decision trees consisted of even more variables than in 1st final classifier 10 variables were typical, see histogram of Figure 8. Variable importance was roughly deduced by overall improvement. The masses used by the 2nd final classifier are listed in Table 3 (starting with most important masses, i.e. masses with highest improvement values). An overview of the distribution of masses of the 2nd final classifier is given in Figure 9.

The 3rd final classifier for colon cancer employed 80 masses out of 90 determined signal clusters. Single decision trees consisted of even more variables than in 1st final classifier 10 variables were typical, see histogram of Figure 10. Variable importance was roughly deduced by overall improvement. The masses used by the 3rd final classifier are listed in Table 4 (starting with most important masses, i.e. masses with highest improvement values). An overview of the distribution of masses of the 3rd final classifier is given in Figure 11.

With the exception of mass 10722 Da, the classifiers include all of the differentially expressed biomolecules found in this study.

Example 6: Classifying performance

Classification performance is determined for the proof-of-principle classifier on the colon cancer versus endoscopy control data set as well as cross-specific control consisting of presumably healthy blood donors. The classifier achieved 95% sensitivity and 94% specificity on the cancer versus endoscopy control test data set and 94% specificity on 77 samples of blood donors.

For the three final classifiers, we determined their specificity on 77 samples of blood donors. We obtained 92% specificity for the 1st final classifier, 100% specificity for the 2nd final classifier and 97% specificity for the 3rd final classifier.

Table 1: Ranking of masses of proof-of-principle classifier by overall improvement.

mass	improvement	mass	improvement	mass	improvement
5493	11.397	6447	0.193	11465	0.048
4964	0.915	15879	0.193	8703	0.046
6645	0.724	4719	0.188	13290	0.045
12619	0.519	3228	0.176	4507	0.041
8781	0.511	17263	0.17	3457	0.04
3947	0.483	15005	0.159	8215	0.039
7576	0.464	17617	0.157	5027	0.038
10595	0.446	2509	0.155	9860	0.038
22952	0.442	9078	0.153	5113	0.031
6852	0.415	4104	0.132	4295	0.03
3327	0.409	13633	0.127	17890	0.028
22467	0.405	7000	0.122	11694	0.027
24080	0.398	2733	0.105	11908	0.026
3021	0.359	9202	0.095	4546	0.025
13829	0.346	16104	0.086	16164	0.025
8375	0.342	18116	0.082	9642	0.014
2270	0.323	9718	0.08	22339	0.013
9143	0.267	4242	0.069	13957	0.012
4866	0.229	6898	0.067	4830	0.011
4339	0.225	4476	0.066	5854	0.011
2949	0.223	8922	0.066	5773	0.009
8077	0.214	7658	0.062		
13784	0.202	8474	0.058		
22677	0.202	12470	0.058		
17397	0.198	5648	0.052		

Table 2: Ranking of masses of 1st final classifier by overall improvement.

mass	improvement	mass	improvement	mass	improvement
5493	12.849	17890	0.157	3947	0.056
6645	1.216	10595	0.156	2733	0.051
4964	0.907	7658	0.148	9581	0.046
8781	0.559	11216	0.147	28259	0.045
12829	0.494	2509	0.141	4607	0.044
15879	0.392	3228	0.141	4546	0.042
2021	0.363	16105	0.128	9930	0.039
22952	0.353	22467	0.112	17617	0.039
2270	0.323	9360	0.111	3457	0.038
28055	0.305	4476	0.099	22677	0.036
18116	0.3	4830	0.093	13633	0.033
8077	0.298	9143	0.088	11694	0.032
6852	0.268	10369	0.088	11905	0.031
2049	0.252	17767	0.085	8703	0.028
4359	0.239	4242	0.083	11465	0.024
8575	0.233	6447	0.078	13983	0.024
24080	0.232	22339	0.078	9078	0.022
12619	0.197	15005	0.075	14798	0.022
7576	0.179	4719	0.073	16953	0.021
12470	0.168	7000	0.064	13290	0.021
4104	0.166	5113	0.062	11547	0.02
15957	0.165	9202	0.062	5648	0.011
17263	0.165	4866	0.058	5226	0.01
5854	0.161	16164	0.058	6898	0.01
3327	0.161	3027	0.057	5773	0.009

Table 3: Ranking of masses of 2nd final classifier by overall improvement.

mass	improvement	mass	improvement	mass	improvement
3947	5.672	9360	0.187	8575	0.068
12829	2.203	3027	0.179	10369	0.066
6645	1.472	4866	0.169	17767	0.063
4964	1.441	12470	0.163	15350	0.056
8077	1.158	9078	0.148	11216	0.046
28055	1.072	2509	0.147	17890	0.044
15957	0.912	6898	0.142	8703	0.039
6852	0.811	10595	0.139	4295	0.036
12619	0.539	7576	0.135	15005	0.036
24080	0.393	8781	0.116	22677	0.036
3327	0.385	22339	0.115	9581	0.031
28259	0.34	5854	0.114	9426	0.03
2021	0.337	2270	0.11	13290	0.027
16105	0.316	6447	0.106	15879	0.026
11694	0.315	22952	0.104	17397	0.023
4104	0.299	4242	0.092	5648	0.022
2049	0.293	10215	0.092	17617	0.022
4719	0.27	5113	0.09	8474	0.019
16164	0.25	9202	0.089	10440	0.016
3457	0.241	9143	0.086	4359	0.009
4546	0.238	13983	0.082	5226	0.008
17263	0.232	4830	0.081	7000	0.006
16953	0.228	4476	0.08	7658	0.006
2733	0.225	11465	0.072		
22467	0.218	18116	0.071		
5773	0.193	15140	0.07		
3228	0.19	4607	0.068		

Table 4: Ranking of masses of 3rd final classifier by overall improvement.

mass	improvement	mass	improvement	mass	improvement
4964	0.431	10595	0.197	18189	0.047
12829	0.266	17658	0.183	7000	0.046
6645	0.259	9078	0.173	22467	0.044
21053	0.248	8781	0.171	10569	0.042
28259	0.152	5773	0.144	18390	0.042
6892	0.089	2870	0.134	15250	0.041
5327	0.081	5115	0.133	6898	0.038
16105	0.077	7570	0.132	17767	0.038
14953	0.076	9143	0.131	8703	0.036
13957	0.074	8447	0.128	13633	0.036
12619	0.068	2793	0.111	15009	0.036
8877	0.066	18116	0.109	15330	0.032
4830	0.062	4607	0.104	13784	0.031
4546	0.055	11694	0.104	17617	0.029
2021	0.053	15879	0.1	14798	0.027
4842	0.049	9902	0.099	17397	0.026
4719	0.044	10215	0.092	5226	0.026
12470	0.042	4476	0.089	9426	0.025
9560	0.038	9581	0.089	5648	0.022
3457	0.037	11905	0.086	8474	0.019
28982	0.035	4339	0.079	8573	0.019
2509	0.031	4295	0.075	16440	0.016
4104	0.025	4466	0.068	17263	0.009
2069	0.23	9718	0.068	11216	0.008
24089	0.219	11465	0.062		
16164	0.201	13983	0.062		
3228	0.198	22339	0.056		
3854	0.192	3027	0.047		

We claim:

1. A method for the differential diagnosis of a colorectal cancer and/or a non-malignant disease of the large intestine, *in vitro*, comprising:
   a) obtaining a test sample from a subject,
   b) contacting test sample with a biologically active surface under specific binding conditions
   c) allowing the biomolecules within the test sample to bind said biologically active surface,
   d) detecting bound biomolecules using a detection method, wherein the detection method generates a mass profile of said test sample,
   e) transforming the mass profile into a computer readable form, and
   f) comparing the mass profile of e) with a database containing mass profiles specific for healthy subjects, subjects having a precancerous lesion of the large intestine, subjects having colorectal cancer, subjects having metastasised colorectal cancer, or subjects having a non-malignant disease of the large intestine,

   wherein said comparison allows for the differential diagnosis of a subject as healthy, having a precancerous lesion of the large intestine, having a colorectal cancer, having a metastasised colorectal cancer and/or a non-malignant disease of the large intestine.

2. The method of claim 1, wherein the database is generated by
   a) obtaining biological samples from healthy subjects, subjects having a precancerous lesion of the large intestine, subjects having colorectal cancer, subjects having metastasised colorectal cancer, and subjects having a non-malignant disease of the large intestine,
   b) contacting said biological samples with a biologically active surface under specific binding conditions,
   c) allowing the biomolecules within the biological samples to bind to said biologically active surface,
   d) detecting bound biomolecules using a detection method, wherein the detection method generates mass profiles of said biological samples,
   e) transforming the mass profiles into a computer-readable form,
   f) applying a mathematical algorithm to classify the mass profiles in e) as specific for healthy subjects, subjects having a precancerous lesion of the large intestine, subjects having colorectal cancer, subjects having metastasised colorectal cancer, and subjects having a non-malignant disease of the large intestine.

3. The method of claim 1, wherein the biomolecules are characterized by:
   a) diluting a sample 1:5 in a desalting buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 3% Ampholine, at 0° to 4°
   b) further diluting said sample 1:10 with a binding buffer consisting of 0.1 M Tris-HCl, 0.05% Triton X-100, pH 8.5 at 0° to 4°
   c) contacting the sample with a biologically active surface comprising positively charged quaternary ammonium groups
   d) incubating of the treated sample with said biologically active surface for 120 minutes under temperatures between 20 and 24° C. at pH 8.5;
   e) and analyzing the bound biomolecules by gas phase ion spectrometry.

4. The method of claim 1, wherein the detection method is mass spectrometry.

5. The method of claim 4, wherein the method of mass spectrometry is selected from the group of matrix-assisted laser desorption ionization/time of flight (MALDI-TOF), surface enhanced laser desorption ionization/time of flight (SELDI-TOF), liquid chromatography, MS-MS and/or ESI-MS.

6. The method of claim 1, wherein the biologically active surface comprises an adsorbent selected from the group of quaternary ammonium groups, carboxylate groups, groups with alkyl or aryl chains, groups such as nitrilotriacetic acid for immobilized metal ions, or proteins, antibodies, or nucleic acids.

7. The method of claim 1, wherein the mass profile comprises a panel of one or more differentially expressed biomolecules.

8. The method of claim 7, wherein, wherein the biomolecules are selected from a group having the apparent molecular mass of 2020 Da ± 10 Da, 2049 Da ± 10 Da, 2270 Da ± 11 Da, 2508 Da ± 13 Da, 2732 Da ± 14 Da, 3026 Da ± 15 Da, 3227 Da ± 17 Da, 3326 Da ± 17 Da, 3456 Da ± 17 Da, 3946 Da ± 20 Da, 4108 Da ± 21 Da, 4262 Da ± 21 Da, 4295 Da ± 21 Da, 4339 Da ± 22 Da, 4476 Da ± 22 Da, 4546 Da ± 23 Da, 4607 Da ± 23 Da, 4915 Da ± 24 Da, 4930 Da ± 24 Da, 4963 Da ± 24 Da, 4983 Da ± 25 Da, 5112 Da ± 26 Da, 5224 Da ± 26 Da, 5495 Da ± 27 Da, 5648 Da ± 28 Da, 5772 Da ± 29 Da, 5934 Da ± 29 Da, 6436 Da ± 32 Da, 6644 Da ± 33 Da, 6852 Da ± 34 Da, 6897 Da ± 34 Da, 6939 Da ± 35 Da, 7575 Da ± 38 Da, 7637 Da ± 38 Da, 8076 Da ± 40 Da, 8215 Da ± 41 Da, 8474 Da ± 42 Da, 8574 Da ± 43 Da, 8702 Da ± 44 Da, 8780 Da ± 44 Da, 8922 Da ± 45 Da, 9078 Da ± 45 Da, 9143 Da ± 46 Da, 9201 Da ± 46 Da, 9339 Da ± 47 Da, 9425 Da ± 47 Da, 9781 Da ± 49 Da, 9641 Da ± 48 Da, 9718

Da ± 49 Da, 9930 Da ± 50 Da, 10215 Da ± 51 Da, 10369 Da ± 52 Da, 10440 Da ± 52 Da, 10594 Da ± 53 Da, 11216 Da ± 56 Da, 11464 Da ± 57 Da, 11547 Da ± 58 Da, 11693 Da ± 58 Da, 11905 Da ± 60 Da, 12470 Da ± 62 Da, 12619 Da ± 63 Da, 12828 Da ± 64 Da, 13290 Da ± 66 Da, 13632 Da ± 68 Da, 13784 Da ± 69 Da, 13983 Da ± 70 Da, 14798 Da ± 74 Da, 15005 Da ± 75 Da, 15140 Da ± 76 Da, 15350 Da ± 77 Da, 15879 Da ± 79 Da, 15957 Da ± 80 Da, 16104 Da ± 81 Da, 16164 Da ± 81 Da, 16953 Da ± 85 Da, 17263 Da ± 86 Da, 17397 Da ± 87 Da, 17617 Da ± 88 Da, 17766 Da ± 89 Da, 17890 Da ± 89 Da, 18115 Da ± 91 Da, 18390 Da ± 92 Da, 22538 Da ± 112 Da, 22466 Da ± 112 Da, 22676 Da ± 113 Da, 22951 Da ± 115 Da, 24079 Da ± 120 Da, 28055 Da ± 140 Da and/or 28259 Da ± 141 Da.

9. A method for the identification of differentially expressed biomolecules wherein the biomolecules of any of claims 1-8 are proteins, comprising:
   a) chromatography and fractionation,
   b) analysis of fractions for the presence of said differentially expressed proteins and/or fragments thereof, using a biologically active surface,
   c) further analysis using mass spectrometry to obtain amino acid sequences encoding said proteins and/or fragments thereof, and
   d) searching amino acid sequence databases of known proteins to identify said differentially expressed proteins by amino acid sequence comparison.

10. The method of claim 9, wherein the method of chromatography is selected from high performance liquid chromatography (HPLC) or fast protein liquid chromatography (FPLC).

11. The method of claim 9, wherein the mass spectrometry used is selected from the group of matrix-assisted laser desorption ionization/time of flight (MALDI-TOF), surface enhanced laser desorption ionization/time of flight (SELDI-TOF), liquid chromatography, MS-MS and/or ESI-MS.

12. A method for the differential diagnosis of a colorectal cancer and/or a non-malignant disease of the large intestine, *in vitro*, comprising detection of one or more differentially expressed biomolecules wherein the biomolecules are polypeptides, comprising:
   a) obtaining a test sample from a subject,
   b) contacting said sample with a binding molecule specific for a differentially expressed polypeptide identified in claims 9-11,
   c) detecting the presence or absence of said polypeptide(s),
   wherein the presence or absence of said polypeptide(s) allows for the differential diagnosis of a subject as healthy, having a precancerous lesion of the large intestine,

having a colorectal cancer, having a metastasized colorectal cancer and/or a non-malignant disease of the large intestine.

13. The method of any one of claims 1-12, wherein the colorectal cancer is a cancer of the colon or rectum.

14. The method of any one of claims 1-12, wherein the test sample is a blood, blood serum, plasma, nipple aspirate, urine, semen, seminal fluid, seminal plasma, prostatic fluid, extracts, tears, saliva, sweat, biopsy, ascites, cerebrospinal fluid, milk, lymph, or tissue extract sample.

15. The method of any one of claims 1-12, wherein the biological sample is a blood, blood serum, plasma, nipple aspirate, urine, semen, seminal fluid, seminal plasma, prostatic fluid, extracts, tears, saliva, sweat, biopsy, ascites, cerebrospinal fluid, milk, lymph, or tissue extract sample.

16. The method or kit of any one of claims 1-12, wherein the subject is of mammalian origin.

17. The method of claim 16, wherein the subject is of human origin.

18. A kit for the diagnosis of a colorectal cancer or a non-malignant disease of the large intestine using the method of any one of claims 1-11 and 13-17 comprising a desorption solution, a binding solution, a washing solution, a biologically active surface comprising an adsorbent, and instructions to use the kit.

19. A kit for the diagnosis of a colorectal cancer or a non-malignant disease of the large intestine using the method of any one of claims 12-17 comprising a solution, binding molecule, detection reagents, and instructions to use the kit.

Figure 1

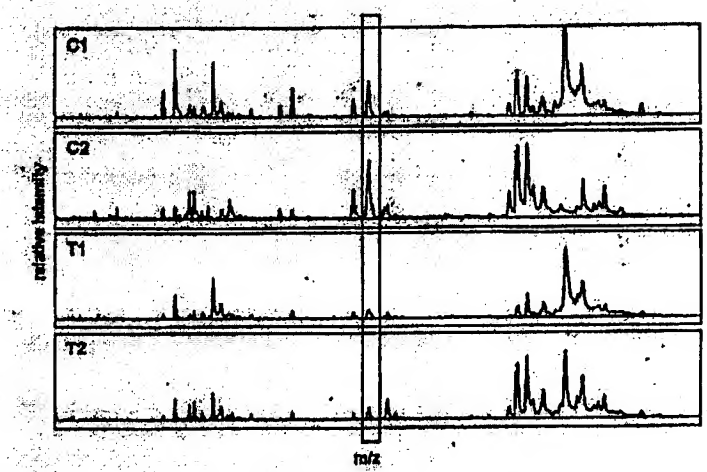


Figure 2A

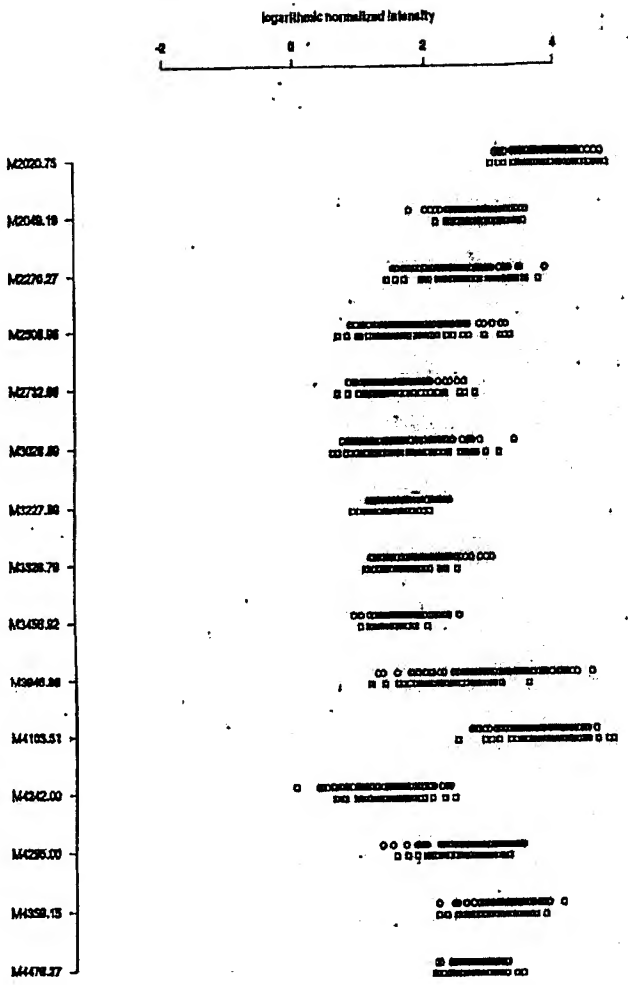


Figure 2B

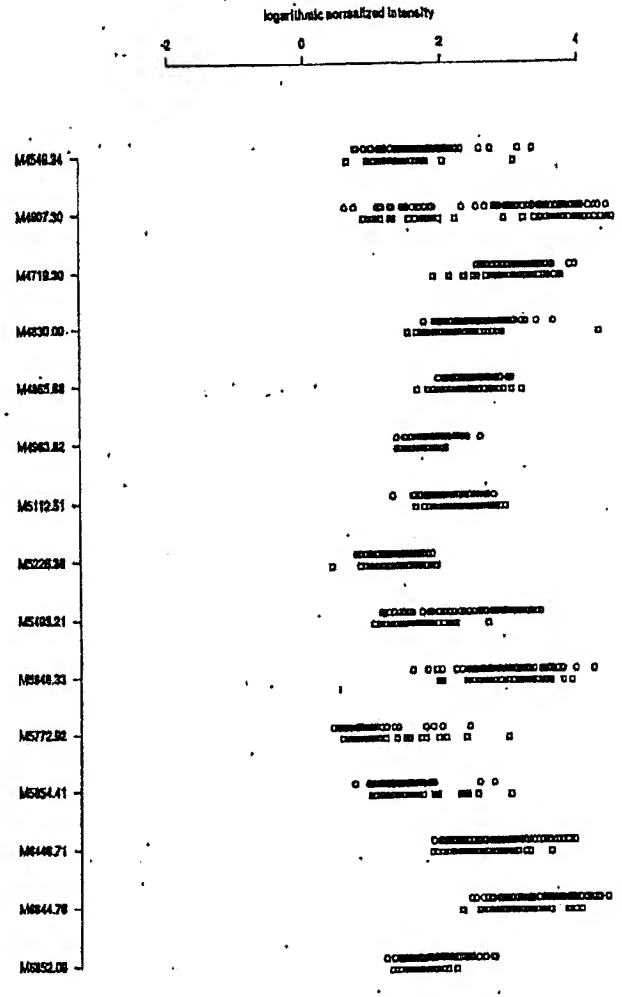
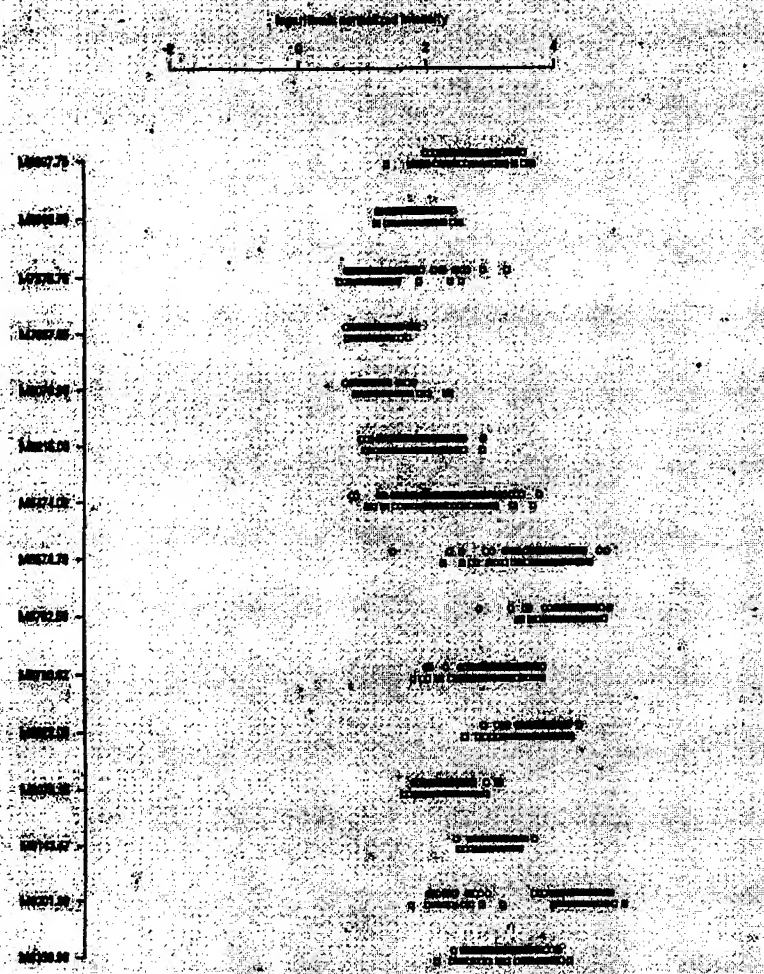


Figure 2C



WO 2004/102199 PCT/EP2004/005394

Figure 2D

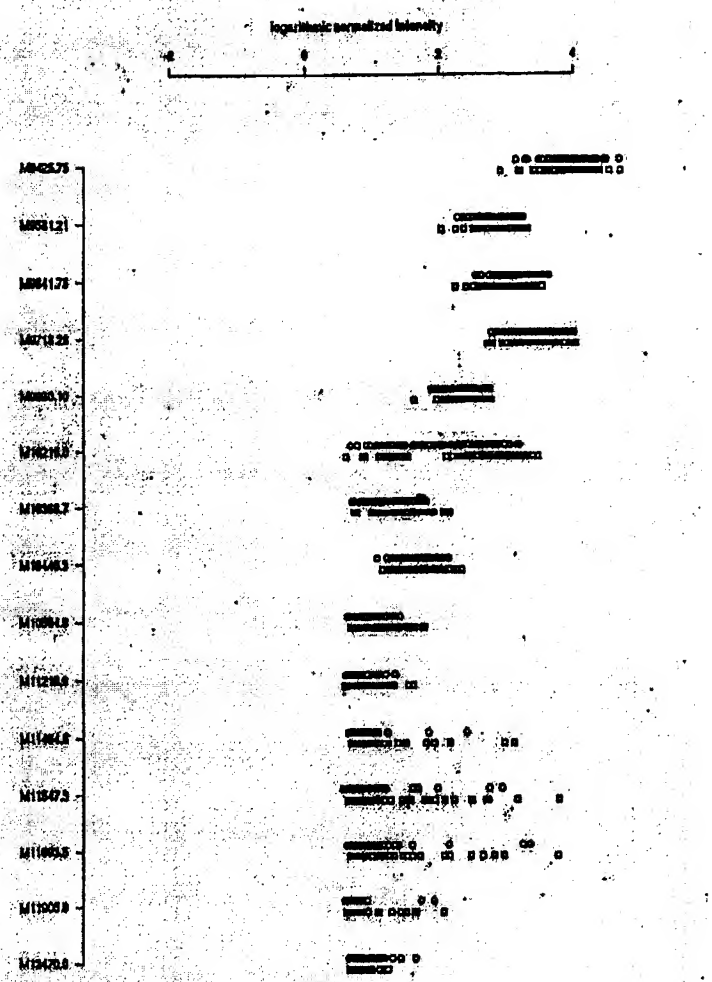


Figure 2E

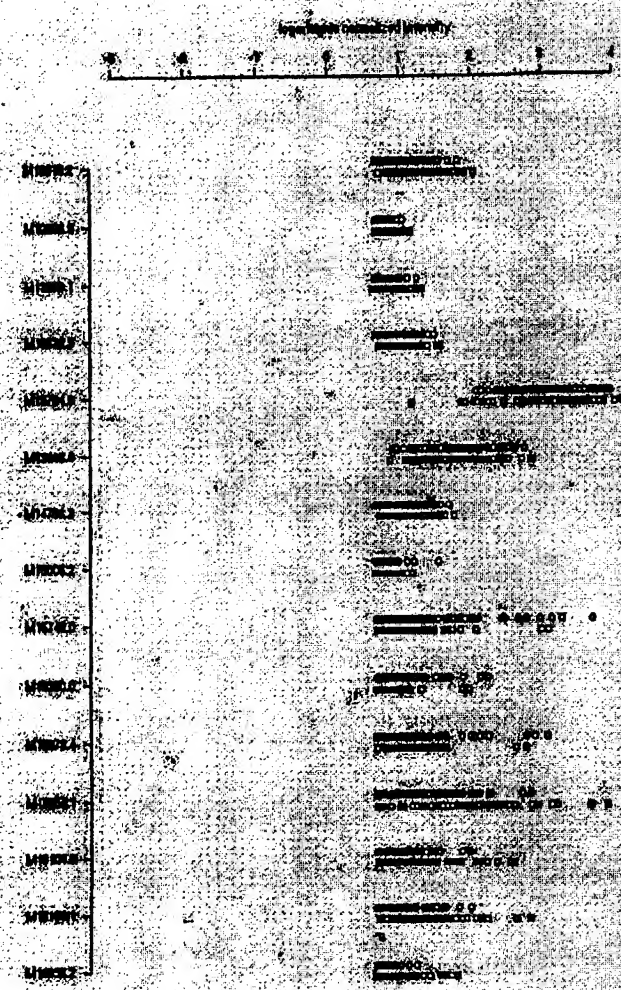


Figure 2F

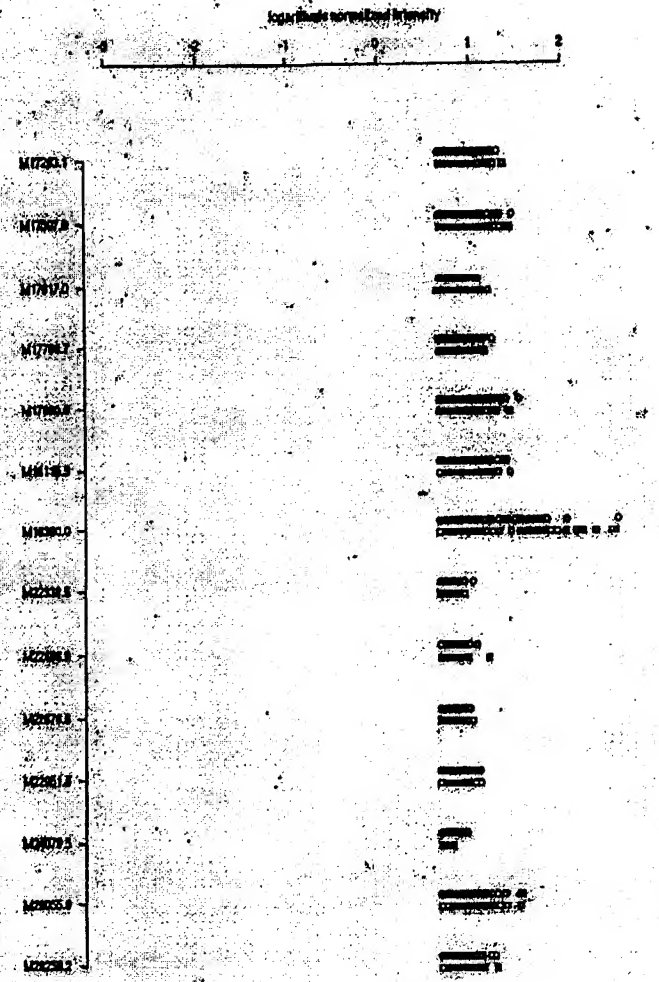


Figure 3A

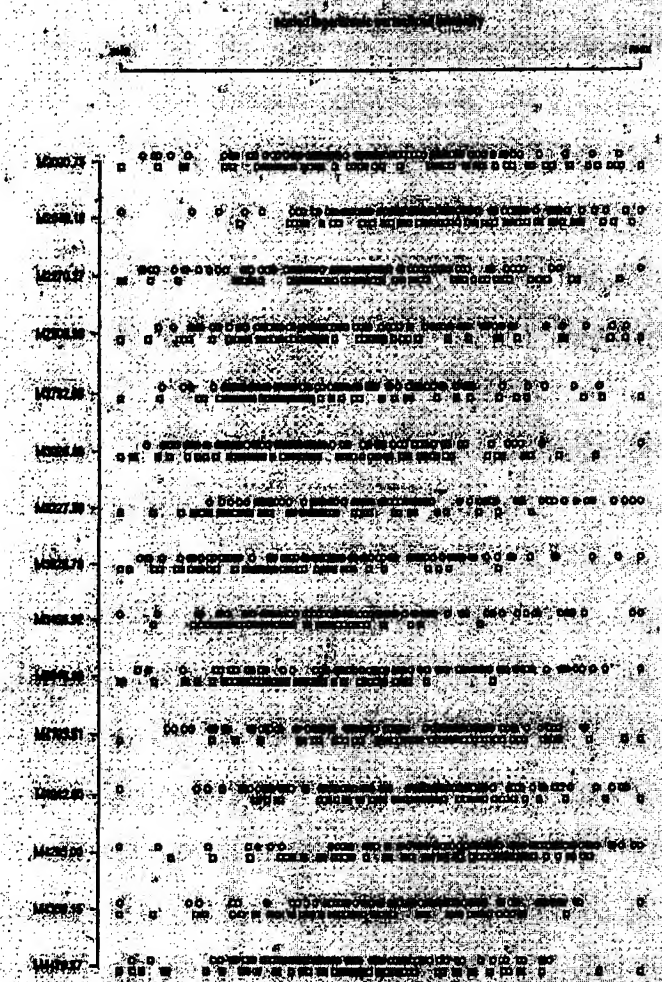


Figure 3B

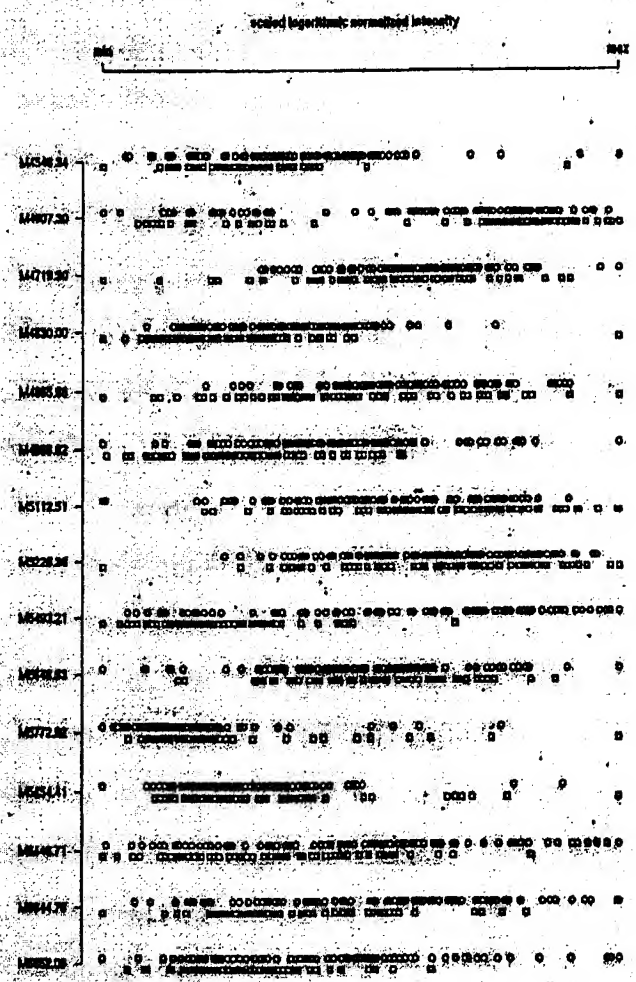


Figure 3C

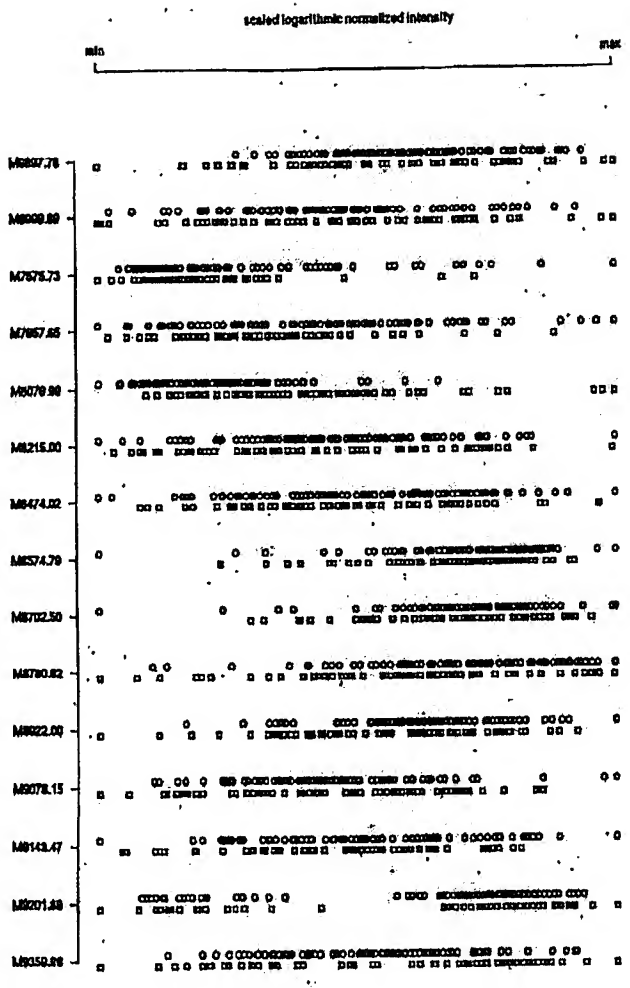


Figure 3D

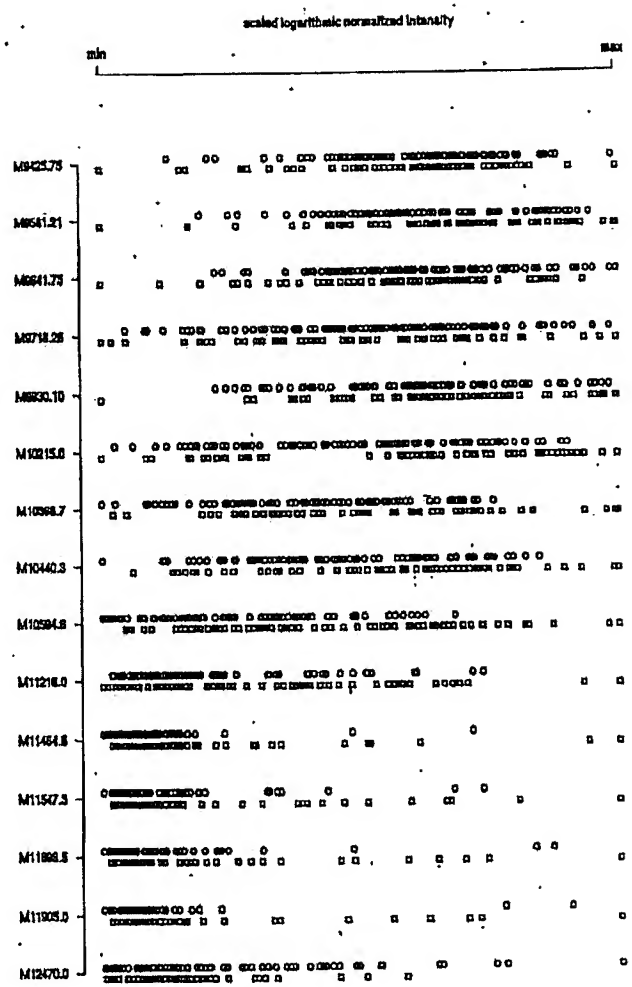


Figure 3E

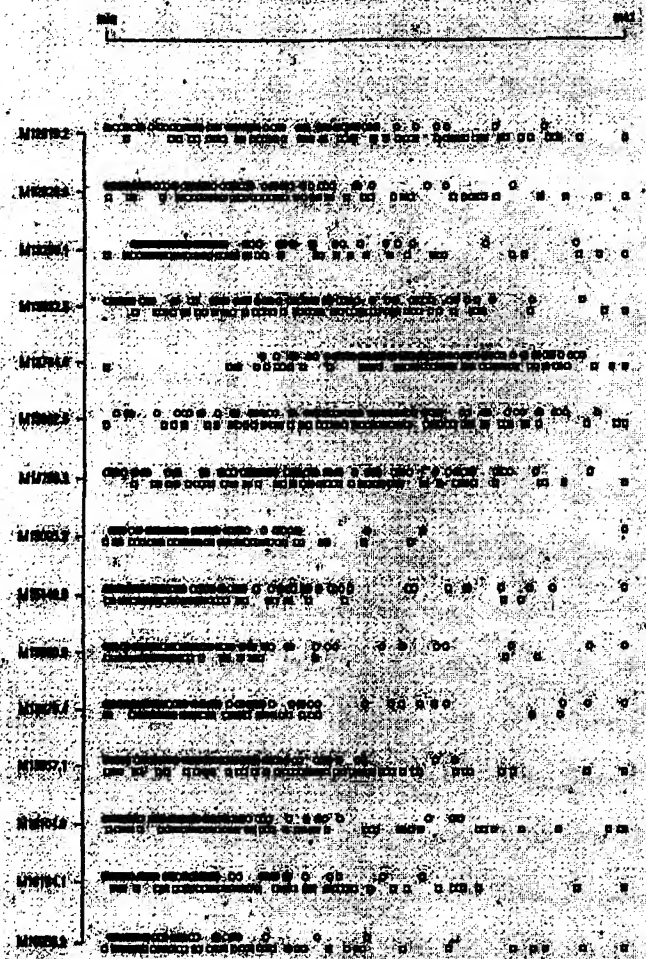


Figure 3F

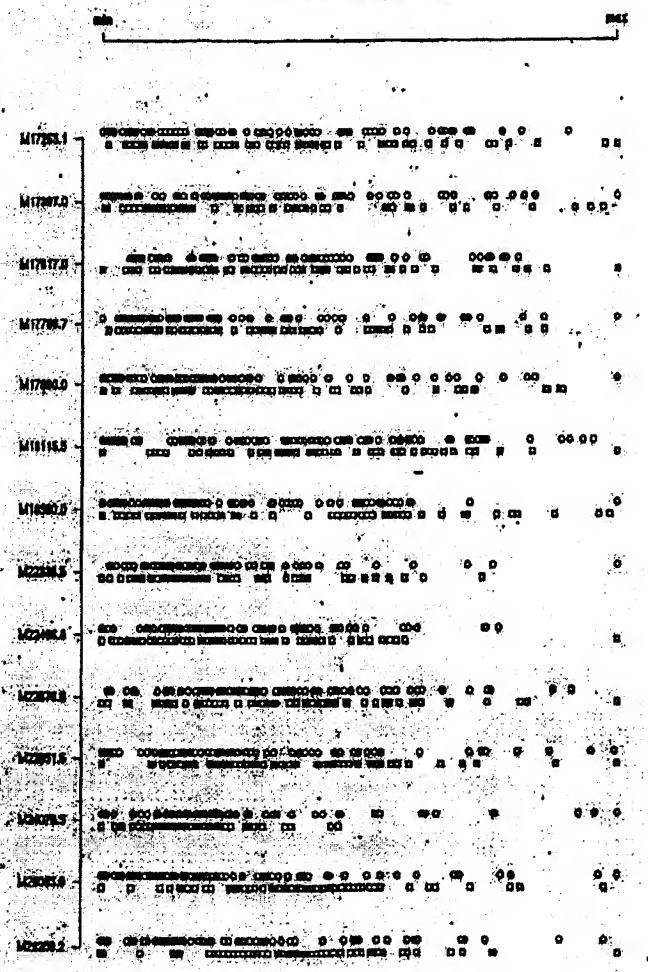


Figure 4

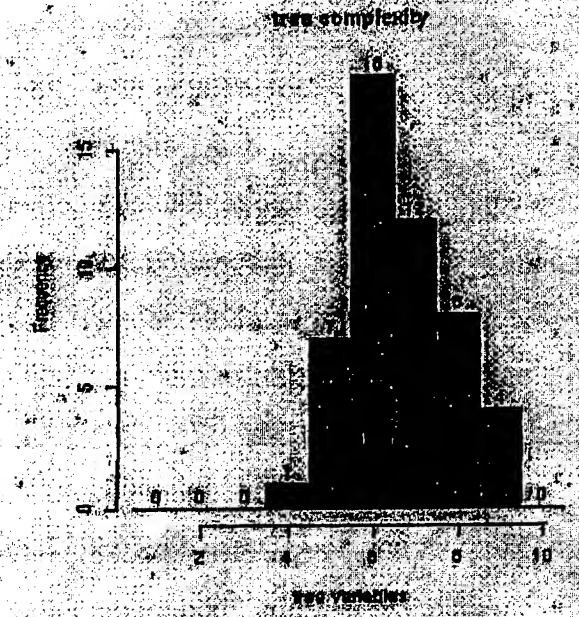


Figure 5

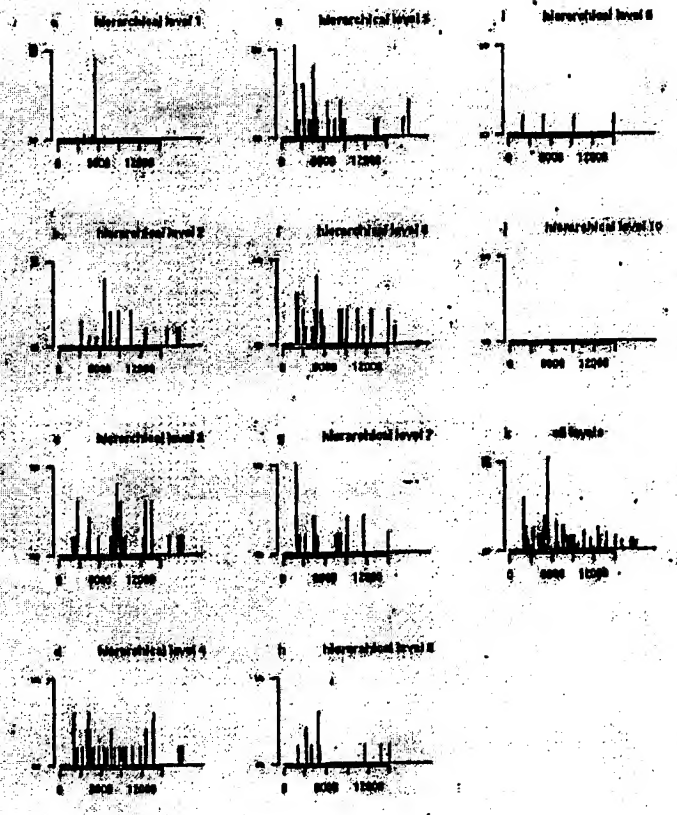


Figure 6

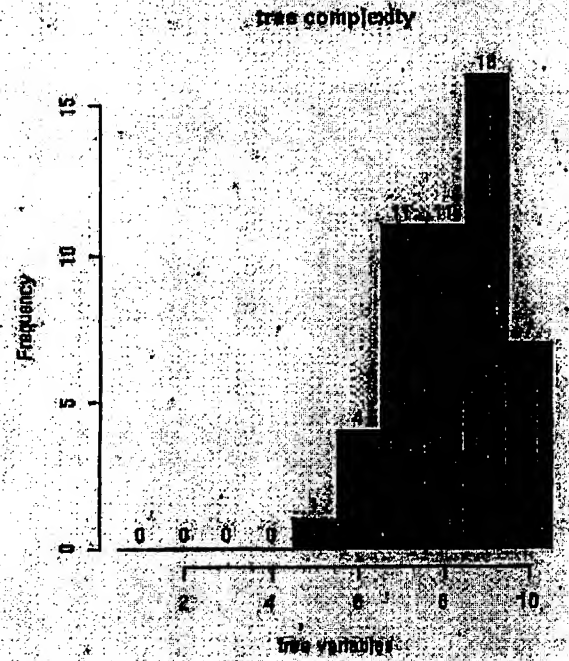


Figure 7

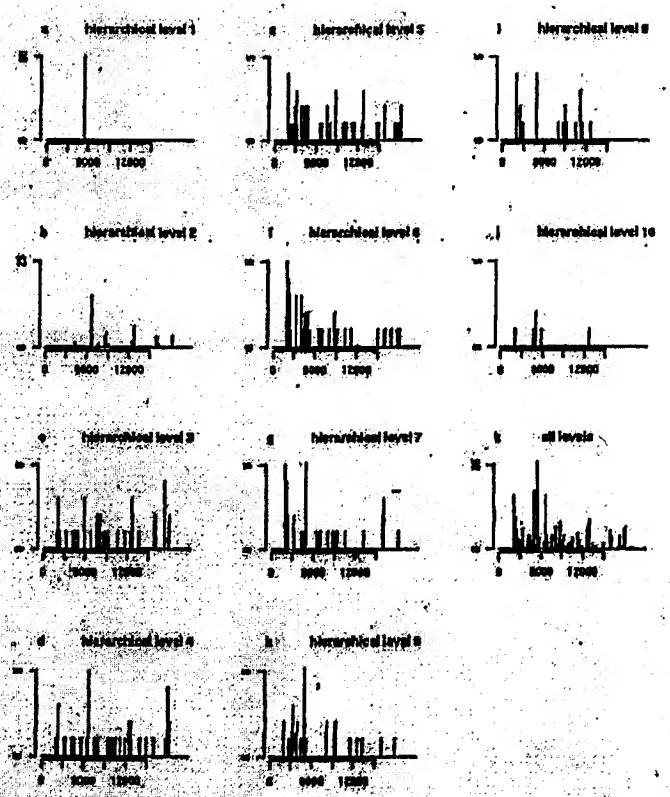


Figure 8

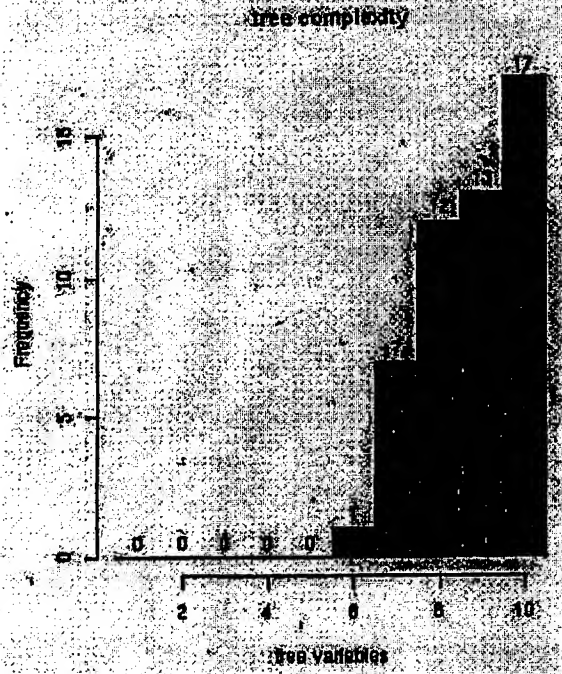


Figure 9

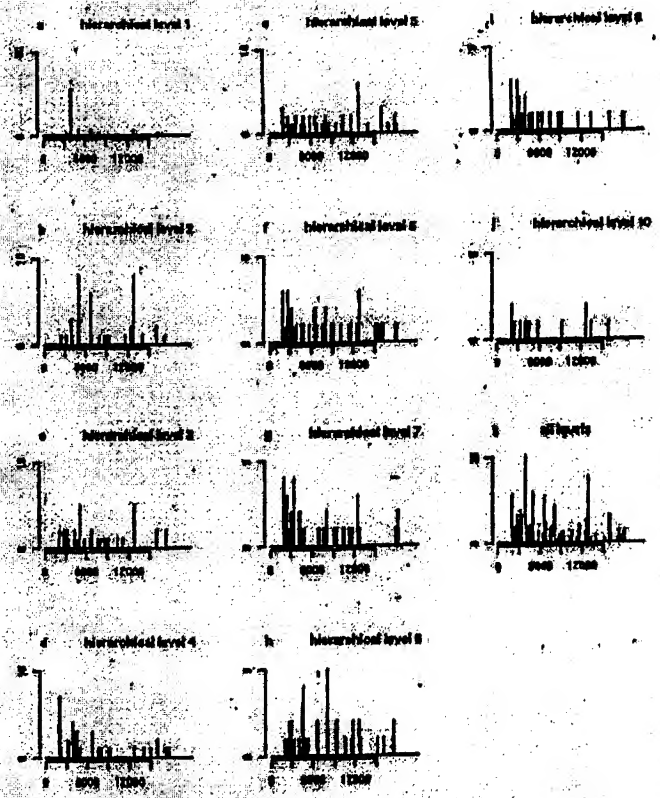


Figure 10

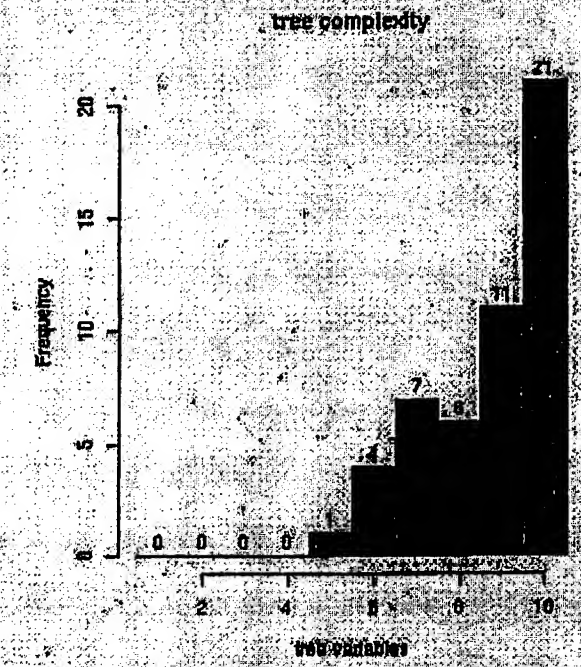
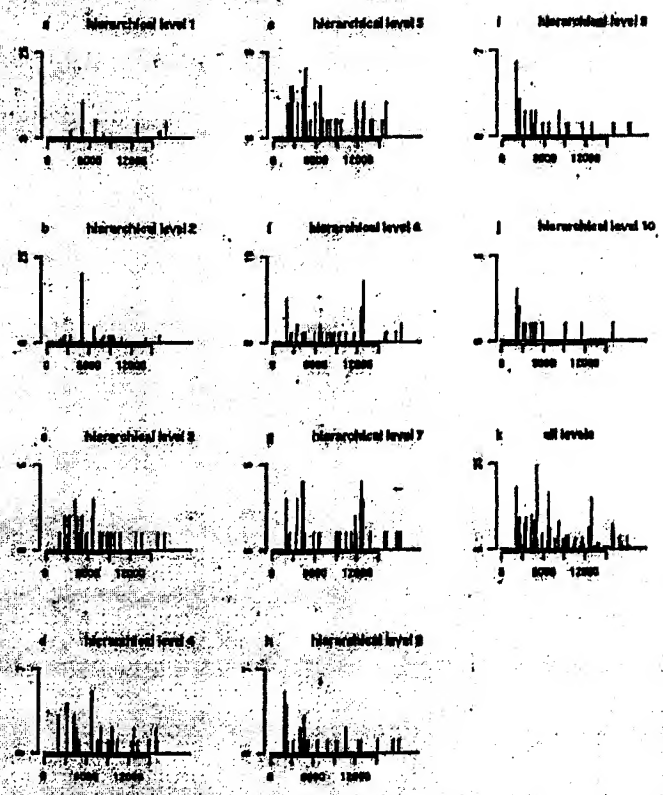


Figure 11



INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/005294

IPC 7 G01N33/48 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/23200 A (YIP CHRISTINE ; DOERFLINGER (US); KITAGAWA NORTON (US); PHAN THANG T (US)) 21 March 2002 (2002-03-21) page 4, line 7 - line 21	1-19
X	SRINIVAS POTHUR R ET AL: "Proteomics in early detection of cancer" CLINICAL CHEMISTRY, vol. 47, no. 10, October 2001 (2001-10), pages 1901-1911, XP002290258 ISSN: 0009-9147 page 1903, column 2 - page 1906, column 1, line 2	1-19

Further documents are listed in the continuation of Box C.

Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 July 2004	16/08/2004
Name and mailing address of the ISA	Authorized officer
	Marchesini, P

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/005294

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ISSAQ H J ET AL: "The SELDI-TOF MS approach to proteomics: Protein profiling and biomarker identification" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 2002 UNITED STATES, vol. 292, no. 3, 2002, pages 587-592, XP002290259 ISSN: 0006-291X page 592, paragraph CONCLUSION	1-19
Y	JUNGBLUT P R ET AL: "PROTEOMICS IN HUMAN DISEASE: CANCER, HEART AND INFECTIOUS DISEASES" ELECTROPHORESIS, WEINHEIM, DE, vol. 20, no. 10, July 1999 (1999-07), pages 2100-2110, XP000870393 ISSN: 0173-0835 page 2101, column 1, paragraph 2	1-19
Y	WULFKUHLE J D ET AL: "Proteomic approaches to the diagnosis, treatment, and monitoring of cancer" ZDOI: ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY 2003 UNITED STATES, VOL. 532, PAGE(S) 59-68 , XP002290260 ISSN: 0065-2598 abstract	1-19
A	JI H ET AL: "A TWO-DIMENSIONAL GEL DATABASE OF HUMAN COLON CARCINOMA PROTEINS" ELECTROPHORESIS, WEINHEIM, DE, vol. 18, no. 3/4, 16 September 1996 (1996-09-16), pages 605-611, XP002071493 ISSN: 0173-0835 the whole document	1-19

Form PCT/ISA/210 (continuation of second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/005294

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0223200 A	21-03-2002	AU 8892101 A	26-03-2002
		WO 0223200 A2	21-03-2002

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☑ FADED TEXT OR DRAWING
- ☑ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.